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Combining molecular analyses with predictive modelling to study the population history of Iberian cryptic bats

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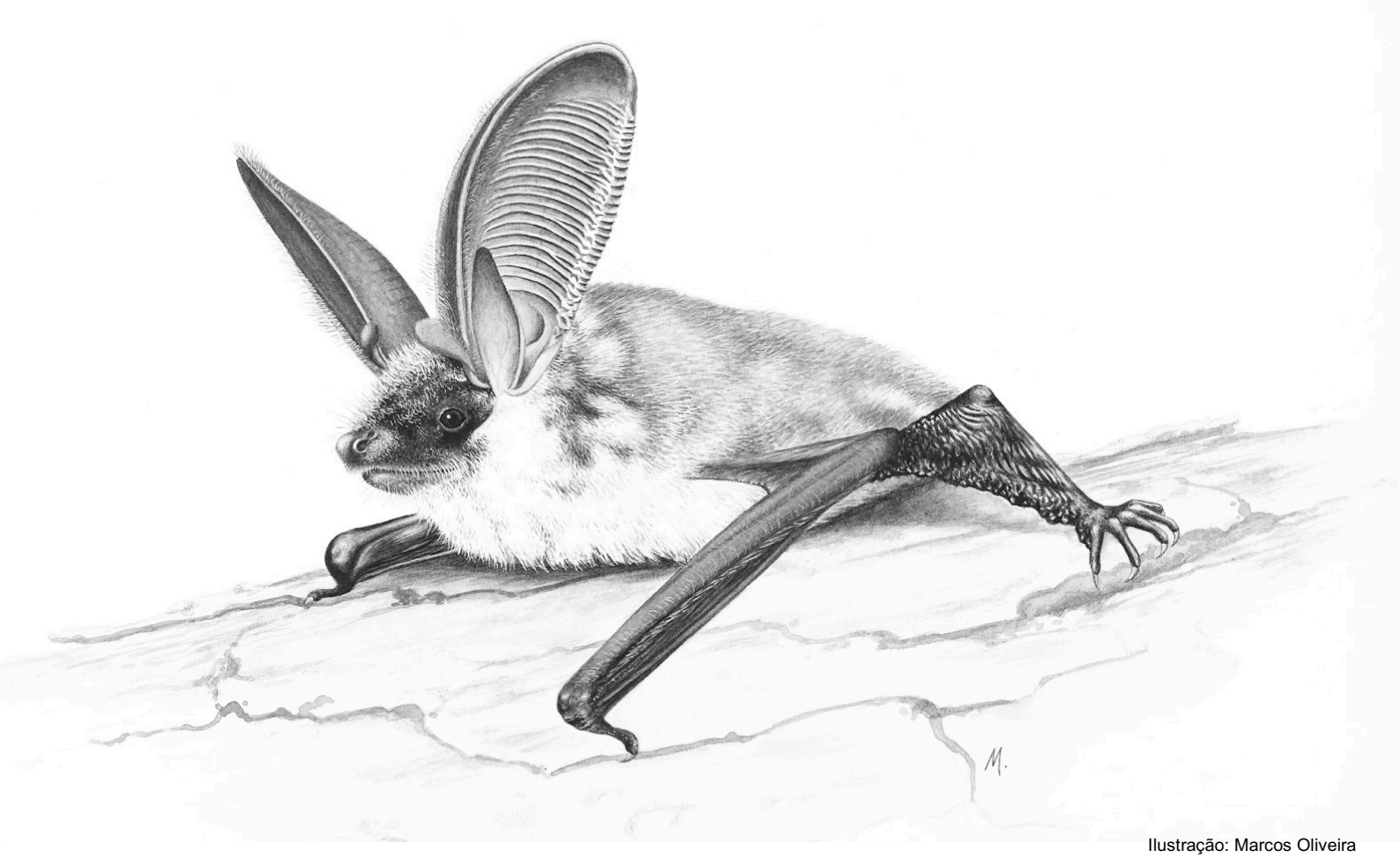

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“There is nothing like looking, if you want to find something.
You certainly usually find something, if you look,
but it is not always quite the something you were after.”
J.R.R. Tolkien, The Hobbit

NOTA PRÉVIA

Na elaboração desta dissertação, e nos termos do número 2 do Artigo 4o do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31o do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada.

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ABSTRACT

Due to the global trends in biodiversity loss, there is an increasing need in conservation biology for the development of methodologies to aid in the fast development of conservation management guidelines. This work focuses on combining methodologies from conservation and landscape genetics with predictive modelling and connectivity, to provide relevant insights on the ecological and molecular characteristics of bat cryptic species, while presenting significant outputs for conservation management. Using the Iberian Peninsula as a case study, this research firstly aimed at determining the distributions, as well as ecological and biogeographical affinities, of recently discovered bat cryptic species. These outputs revealed possible unsurveyed areas of species occurrence as well as the ecological relationships between species' niches and the contact zones within each cryptic complex. With a particular focus on the poorly-known cryptic lineage *Plecotus auritus begognae*, a population evolution study was also developed to clarify the evolutionary history and relationships between the Iberian *Plecotus* lineages. These results revealed a clear separation of all lineages within this genus, except between *Plecotus auritus auritus* and *Plecotus auritus begognae*, which were only differentiated by mitochondrial DNA and microsatellites, therefore suggesting a recent separation and supporting the subspecies status. Successful field campaigns were then conducted in the previously determined subspecies' area of occurrence, with the aim of filling the sampling gaps. Subsequently, a multi-disciplinary approach was developed, integrating landscape genetics' analyses with species distribution modelling and connectivity methodologies, to study the environmental drivers affecting the population structure and connectivity patterns of *P. a. begognae*. This subspecies showed no clear population structure within Iberia, with gene flow occurring throughout its range. The outputs of this study, however, not only demonstrated that this gene flow is dependent on the presence of forested and mountainous areas, but also identified the subspecies' distribution patches which are potentially at-risk of isolation. The large extents of mature forests in the North of the Iberian Peninsula seem to be preventing the fragmentation within this subspecies and, as such, conservation efforts directed to forest preservation in these areas are recommended. Finally, with the aim of evaluating the potential impacts of climate change in *P. a. begognae*'s populations, landscape genetics was integrated with climatic modelling. The results obtained showed a considerable contraction of *P. a. begognae*'s suitable area of occurrence for 2050 and even more severe for 2070. Most of the subspecies' distribution range will become unsuitable, forcing populations to shift their ranges into projected suitable and stable areas, mostly located in north-western Iberia. The most likely pathways for range shift and dispersal, and the climatic predictors most related to them were also determined. Predictions also

revealed possible refuge areas, as some regions located within the western distribution of the subspecies seem to remain stable throughout the decades in climate change scenarios. Overall, the integration of different disciplines proved successful in providing relevant ecological and molecular information for *P. a. begognae*, as well as presenting spatially explicit outputs essential to prioritize conservation measures.

Keywords: Biodiversity conservation, molecular ecology, spatial ecology, landscape genetics, connectivity analyses, climate change, bats, cryptic species.

RESUMO

As tendências globais associadas à perda da biodiversidade têm motivado o desenvolvimento de metodologias para apoiar a gestão na biologia da conservação. O presente trabalho combina metodologias derivadas da genética da conservação e da paisagem, com análises de modelação preditiva e de conectividade, contribuindo para o conhecimento das características ecológicas e moleculares de espécies crípticas de morcegos, e respetiva gestão. Considerando a Península Ibérica como caso de estudo, este trabalho primeiramente focou-se na identificação das distribuições e das afinidades ecológicas e biogeográficas de espécies crípticas de morcegos recentemente descobertas. Esta identificação permitiu reconhecer potenciais áreas de ocorrência das espécies - até então não amostradas -, bem como relações ecológicas entre os nichos das espécies, e zonas de contacto associadas a cada complexo críptico. Com especial atenção sobre a linhagem críptica e pouco estudada, *Plecotus auritus begognae*, foi também desenvolvido um estudo evolutivo de populações para clarificar a história e relações evolutivas entre as linhagens Ibéricas do género *Plecotus*. Os resultados deste estudo revelaram uma separação clara entre todas as linhagens pertencentes a este género, exceto entre *Plecotus auritus auritus* e *Plecotus auritus begognae*. A diferenciação entre estas linhagens foi apenas demonstrada através de DNA mitocondrial e microssatélites, sugerindo uma separação recente e dando suporte ao estatuto de subespécie de *P. a. begognae*. Com o objetivo de colmatar lacunas na amostragem, foram subsequentemente realizadas campanhas de campo nas áreas de ocorrência das subespécies previamente identificadas. Uma abordagem multidisciplinar foi desenvolvida posteriormente para estudar os fatores ambientais que moldam a estrutura populacional e os padrões de conectividade de *P. a. begognae*. Para isso foram integradas análises de genética da paisagem com metodologias de modelação de distribuição de espécies e análises de conectividade. A subespécie mostrou uma estrutura populacional clara, com fluxo génico a ocorrer ao longo de todo a sua distribuição. No entanto, este estudo demonstrou que o os padrões de fluxo génico estão dependentes da presença de manchas florestais e de zonas montanhosas, permitindo a identificação de áreas em potencial risco de isolamento. A extensão de florestas no Norte da Península Ibérica demonstrou ter um papel na prevenção da fragmentação da população da subespécie. Desta forma, sugere-se o direcionamento de esforços de conservação para a preservação de florestas nestas áreas. Finalmente, este trabalho integrou genética da paisagem com modelação climática, com o propósito de avaliar potenciais impactos das alterações climáticas sobre as populações de *P. a. begognae*. Os resultados obtidos mostraram uma redução considerável da área adequada à ocorrência de *P. a. begognae* para o ano de 2050. Esta redução foi ainda mais drástica nas projeções

efetuadas para o ano 2070. Desta forma, grande parte da extensão da distribuição da subespécie poderá vir a tornar-se inadequada, forçando futuras alterações na distribuição e dispersão das populações para áreas mais estáveis e adequadas. As rotas mais prováveis de dispersão e os seus respetivos fatores determinantes foram também identificados. As previsões efetuadas revelaram igualmente áreas potenciais de refúgio, devido ao facto de algumas regiões na distribuição mais oeste da subespécie se manterem estáveis ao longo das décadas futuras de potenciais alterações climáticas. Em suma, a integração das diferentes disciplinas neste trabalho revelou-se bem-sucedida na aquisição de informação ecológica e molecular para *P. a. begognae*, permitindo obter resultados espacialmente explícitos e de elevado valor para apoiar e priorizar medidas de conservação.

Palavras-chave: Conservação da biodiversidade, ecologia molecular, ecologia espacial, genética da paisagem, análises de conectividade, morcegos, espécies crípticas.

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AIC	Akaike information criterion
AUC	area under the curve
CMS	Convention on Migratory Species
COI	cytochrome c oxidase subunit I
CR	control region
cyt b	cytochrome-b
DNA	deoxyribonucleic acid
EGV	ecogeographical variables
ENFA	ecological niche factor analyses
ENM	ecological niche models
ESU	evolutionary significant unit
EUROBATS	Agreement on the Conservation of Populations of European Bats
GAM	generalized additive models
GARP	genetic algorithms for rule set production
GCM	global climate models / general circulation models
GIS	geographic information systems
GLM	generalized linear models
GS	global climate model GISS-E2-R
HE	global climate model HadGEM2-ES
H_E	expected heterozygosity
HL	homozygosity by loci
H_O	observed heterozygosity
HWE	Hardy–Weinberg equilibrium
IN	global climate model INMCM4
JC	Jukes-Cantor model
MARS	multivariate adaptive regression splines
MCMC	Markov chain Monte Carlo
ML	maximum-likelihood
MP	global climate model MPI-ESM-LR
MRDM	multiple regressions on distance matrices
mtDNA	mitochondrial DNA
NA	number of alleles per locus
ND1	NADH dehydrogenase gene I

nDNA	nuclear DNA
NJ	neighbor-joining
PCA	principal components analysis
PCR	polymerase chain reaction
qi	individual membership proportions
RCP	representative concentration pathways
ROC	receiver operating characteristics
rRNA	ribosomal ribonucleic acid
SDM	species distribution models
sPCA	spatial principal components analysis
UNEP	United Nations Environmental Programme

CHAPTER 1

GENERAL INTRODUCTION

1.1. CHALLENGES TO BIODIVERSITY CONSERVATION

According to the United Nations Environment Programme and the Convention on Biological Diversity, the concept of biological diversity broadly refers to the variability among living organisms, measured at the genetic, the species and the ecosystem level (Hawksworth, 1995). Although there are several definitions on biodiversity (e.g. taxonomic diversity, morphological diversity, functional diversity, molecular diversity), it is most commonly used to replace the more clearly defined and long established terms, species diversity and species richness. Biodiversity also comprises the variation of and within ecosystems, including the complexity of its processes and functions (MEA, 2005).

Due to drastic and rapid decreases in global biodiversity, we are currently experiencing the phenomenon of the Sixth Extinction. Current trends on habitat destruction and alteration, have led to increasing threats and extinction of numerous species, many still unbeknownst to us (Leakey & Lewin, 1997; Vié *et al.*, 2009). Many implications of biodiversity loss are unknown, and even though some ecosystems may thrive with fewer or different species, some may collapse. Nevertheless, it has been acknowledged that biodiversity plays a significant role in directly providing goods and services, as well as regulating and modulating ecosystem properties and functions (MEA, 2005; Harrison *et al.*, 2014). It has also been demonstrated that declines in species richness may affect ecosystems productivity, alter biotic and trophic interactions among organisms and affect ecosystem services (Symstad *et al.*, 1998; Balvanera *et al.*, 2006; Tylianakis *et al.*, 2008).

Several recent international policies have been created as a response to the current trends in biodiversity and habitat loss (Habitats Directive, Convention on Biological Diversity). In fact, in the United Nations Convention on Biological Diversity, in their Strategic Plan for Biodiversity 2011-2020, all participant parties proposed to achieve the Aichi Biodiversity Targets and stop biodiversity loss by 2020. The targets are to be met by safeguarding ecosystems, species and genetic diversity and reducing the direct pressures on biodiversity, while promoting sustainability.

Another increasingly alarming threat to biodiversity is climate change. Global average temperatures have already increased by 1.5 degrees Celsius during the past century, due to the human activities, mainly through the emission of greenhouse gases. The concentration of carbon dioxide is higher now than it has been at any time in the past 650 000 years (IPCC, 2014). In fact, in 2016, atmospheric carbon dioxide reached an historic threshold, as levels

remained above 400 parts per million, a tipping point for atmospheric carbon dioxide, with potentially heavy implications for future climate conditions (Schmidt, 2017). Consequences of climate change on biodiversity range from changes in the phenology and physiology of organisms and alteration of the composition and interaction within communities, to the complete transformation in structure and dynamics of ecosystems (Walther *et al.*, 2002). One of the most significant consequences of climate change is the shifting of species' distributional ranges, where, due to changes in local conditions, organisms are forced to move from their original areas of occurrence to new suitable regions (Araújo & Rahbek, 2006; IPCC, 2014; Pecl *et al.*, 2017). These tendencies can cause substantial fragmentations within populations and local extinctions (Thomas *et al.*, 2004). This rising need for ecosystem and biodiversity preservation has led to significant developments in the discipline of conservation biology.

Conservation biology is the applied scientific field that addresses the maintenance of Earth's biodiversity, with focus on safeguarding species, communities and ecosystems. It is an interdisciplinary subject combining fields such as biological sciences (e.g. biology, ecology), social sciences (e.g. economics, sociology, education, law), geosciences (e.g. geography, geology) and management sciences (e.g. forestry, fisheries) (Soule & Wilcox, 1980; Soulé, 1985; Hunter Jr & Gibbs, 2006). Its conception and emergence as a new field is attributed to the First International Conference on Conservation Biology, held in 1978, with the intent to bridge the gap between ecological and genetic theories and conservation policies and practices (Soule & Wilcox, 1980).

Nowadays, conservation biology has become a major field, essential for the increasing need in development of conservation policies and measures, and expanded further into several sub disciplines, such as conservation genetics, conservation social science, conservation behaviour and conservation physiology (Hunter Jr & Gibbs, 2006; Sutherland, 2009). In order to maintain biological diversity and ecological integrity and health, conservation requires a complex combination of strategies, including endangered species protection, creation of ecological reserve systems, control of the impacts of human actions in ecological systems, ecosystem restoration, population control by captive breeding, management of harvests, management of non-native species and conservation education (Trombulak *et al.*, 2004).

Furthermore, species conservation involves knowledge on the species itself and its ecosystem, more specifically, its threats and rate of decline, estimations on population structure, sizes and trends, species ecological requirements and its area of geographical distribution (IUCN, 2012). For these purposes, a multitude of techniques have been developed

in the many disciplines within conservation biology, predominantly focusing on the molecular and ecological levels.

1.2 MOLECULAR TOOLS IN CONSERVATION

Genetic diversity is considered one of the fundamental levels of biodiversity, so it is essential in conservation. Studies on the genetic variability of species not only allow for a deeper understanding of species populations' structure and history, but also for the determining of species extinction risk. Population decline is a process directly linked to the loss of intraspecific genetic diversity and is therefore of great importance to understand how the drivers responsible for the decline of biodiversity influence evolutionary processes (Hoffmann & Willi, 2008). Decreased levels of genetic diversity have been associated with reduced fitness, low immunity levels, inbreeding, and population depletion and isolation, all possible drivers of extinction (Lande, 1988; Leberg, 1990; Frankham, 2005). As such, conservation genetics arises as an important discipline, with the aim of aiding in the conservation and restoration of biodiversity (Frankham, 2005), by combining an array of fields such as population genetics, molecular ecology, evolutionary biology, systematics and genomics.

Recent advances in molecular research methods have prompted the development of techniques that contribute significantly to the field of conservation genetics, such as: measurement of phylogenetic diversity (Faith, 1992; Rodrigues & Gaston, 2002), identification of hybridization and introgression (Rhymer & Simberloff, 1996; Mooney & Cleland, 2001), characterization of population structure (Pritchard *et al.*, 2000; Meirmans & Hedrick, 2011), identification of Evolutionary Significant Units (ESUs) and management units for conservation (Legge *et al.*, 1996; Crandall *et al.*, 2000), estimation of population size (Frankham, 1996; Reed, 2005), measurement of the impact of inbreeding and outbreeding depression (Leberg & Firmin, 2008; García-Dorado, 2012), characterization of environmental adaptations (Orr, 2005; Buckley *et al.*, 2010), discovery of new and cryptic species (Bickford *et al.*, 2006; Mayer *et al.*, 2007) and development of new techniques for non-invasive genotyping (Silva *et al.*, 2015; Gulsby *et al.*, 2016).

Several studies have been using molecular methodologies for conservation biology purposes (Rowe *et al.*, 2017), such as the integration of phylogenetic analysis with conservation and assessment of extinction threats (Schachat *et al.*, 2016). Phylogenetics has also been applied in estimating the amount of evolutionary history present in biodiversity hotspots, showing that genetic diversity is considerably higher than species richness alone (Sechrest *et al.*, 2002).

Phylogeography and population genetics have been used, for example, to inform on pollinator's conservation management strategies (López-Uribe *et al.*, 2017) and in identifying priority conservation units of endangered faunal (Taberlet & Bouvet, 1994) and floral (Newton *et al.*, 1999) taxa. Molecular tools have also shown to be valuable in the monitoring of species with management, conservation and ecological interest (Schwartz *et al.*, 2007). More recently, genomic studies have also focused on using single-nucleotide polymorphisms to estimate levels of adaptive variation and divergence, so they can determine species adaptability to conservation threats (van Tienderen *et al.*, 2002; Morin *et al.*, 2004).

However, molecular approaches tend to overlook the spatial characteristics that may influence species genetic structure and variability, such as landscape structure (Storfer *et al.*, 2007). By using genetic methodologies alone, we may be overlooking important processes such as what affects species' gene flow, connectivity, dispersal or other population patterns in heterogeneous and fragmented landscapes, or which are the environmental factors driving species' adaptation processes (Manel & Holderegger, 2013).

1.2 SPATIAL TOOLS IN CONSERVATION

The recent growth of Geographic Information Systems (GIS) and mapping software, with faster and open-source tools, is largely responsible for the growing use of digital mapping methodologies. The use of maps and geographical data are of utmost relevance as analytical and communication tools for researchers and policy shapers in conservation. Since they provide information on species geographic ranges and distributional areas, and on habitat and landscape composition, spatially-explicit methodologies are frequently used in conservation biology.

One of the main approaches using spatial methodologies in conservation is the delimitation of areas relevant for protection, maintenance and connectivity of species and/or habitats. The establishment of protected areas (e.g. nature reserves, national parks, habitat/species management areas, protected landscape/seascapes) is one of the main outputs of conservation biology, and it aims to define a recognised geographical space, dedicated to the management and the long-term preservation of nature (Dudley, 2008). Systematic conservation planning often resorts to spatially-explicit methods in reserve design, with the aim of encompassing or sustaining the highest priority biodiversity values along with the establishment of protective management (Margules & Pressey, 2000). Moreover, the identification of biodiversity hotspots (highly species-rich areas) and coldspots (areas with

poor species richness), one of the main conservation priorities worldwide (Myers *et al.*, 2000; Kareiva & Marvier, 2003), is also highly dependent on spatial data.

Another highly employed spatial methodology in conservation is species distribution modelling (SDMs) (also known as environmental niche modelling, ecological niche modelling or predictive habitat distribution modelling). SDMs refers to the use of statistical or theoretical algorithms that relate field observations with environmental predictors, to produce models of the predicted distribution range of species in geographic space (Guisan & Zimmermann, 2000). The delimitation of species distributions and the prediction of their possible occurrence areas is fundamental for species conservation and population management (Rushton *et al.*, 2004; Pearson *et al.*, 2007). Predictive modelling presents, therefore, as an essential tool, providing insights into species environmental tolerances and habitat preferences, while also quantifying species' environmental niches (Anderson *et al.*, 2003; Elith & Leathwick, 2009). Besides the production of maps of probability of occurrence or habitat suitability, this methodology has shown applicability in a wide variety of fields relevant for conservation, such as: conservation planning, delimitation of protected areas and reserve design (Araújo & Williams, 2000; Ferrier *et al.*, 2007; Carvalho *et al.*, 2010; Beresford *et al.*, 2011); survey planning for rare or undiscovered species and distributional areas (Engler *et al.*, 2004; Pearson *et al.*, 2007); predicting the risk and impacts of invasive species (Peterson, 2003; Jeschke & Strayer, 2008); quantification of the environmental niche of species (Austin *et al.*, 1990; Vetaas, 2002); and assessing impacts of climate change on biodiversity (Araújo *et al.*, 2004; Thomas *et al.*, 2004).

With the advent of their popularity, several different algorithms have been developed for SDMs. These can be mainly characterised by the type of input species data they use, presence-absence or presence-only (Franklin, 2010). If biological surveys allow to record species presence and absence, regression-based techniques are generally used, such as logistic multiple regression (Homser & Lemeshow, 1989; Franco *et al.*, 2000) generalized linear models (GLM; Nelder & Wedderburn, 1972; Guisan & Theurillat, 2000), generalized additive models (GAM; Hastie & Tibshirani, 1990; Fewster *et al.*, 2000) or multivariate adaptive regression splines (MARS; Friedman, 1991; Moisen & Frescino, 2002). However, the use of absence records is not always adequate, as they can be unreliable depending on the focus species. Absence information can be challenging to obtain for species with elusive behaviours and low detectability, resulting in false absence records, i.e. when species could not be detected even though it was present (Hirzel & Le Lay, 2008; Rebelo & Jones, 2010). Therefore, presence-only modelling approaches have been developed, such as maximum

entropy (Maxent; Phillips *et al.*, 2006; Phillips & Dudik, 2008), ecological niche factor analyses (ENFA; Hirzel *et al.*, 2002; Engler *et al.*, 2004), genetic algorithms for rule set production (GARP; Stockwell, 1999; Pearson *et al.*, 2007) or envelope models like BIOCLIM (Beaumont *et al.*, 2005; Booth *et al.*, 2014). Furthermore, predictive model calculations are not restricted to the use of a single algorithm as ensemble models can be created from several algorithm outputs, allowing for the development of consensus models. Consensus models, when produced and analysed appropriately, can present robust forecasts, as they consist in the ensemble of the algorithms that show higher predictive performance (Araújo & New, 2006). The selection of algorithms will, however, depend on factors such as the scale of analyses, species' niche breadth, quantity and accuracy of species data, selection of ecological predictors, dispersal limitations, biotic interactions, among others (Elith *et al.*, 2010).

Nevertheless, the majority of research using spatial methodologies does not take into account the molecular characteristics of target species. Genetic variability, population structure, gene flow, phylogenetic relationships and other molecular-level characteristics are essential insights for more effective conservation planning. Integrating genetic information in spatial analyses is essential to understand how geographical and environmental features influence the genetic variation of species.

1.3 LANDSCAPE GENETICS

Due to the growing interest in the integration of molecular analyses with spatially-explicit information, several research fields have been focusing on this multi-disciplinary task, particularly in biogeographic and phylogeographic studies (Avise, 2000; Hugall *et al.*, 2002; Queiroz, 2005; Kidd & Ritchie, 2006; Richards *et al.*, 2007; Morrone, 2008). However, one field is particularly successful in assessing the interaction between geographic and molecular features, as well as presenting conservation relevant results, and that is the recently emergent field of landscape genetics.

Landscape genetics is the study of how landscape influences the genetic variability, structure and gene flow of populations across space and time, and on how landscape features influence spatial genetic variation (Manel *et al.*, 2003). It is an interdisciplinary field, combining the tools of molecular and population genetics with the principles and concepts of landscape ecology (Holderegger & Wagner, 2008). The integration of spatial and genetic information builds a bridge between ecological biogeography and molecular ecology, allowing for a better

understanding of how geographical and environmental features of the landscape affect genetic variation, population structure and gene flow (Manel *et al.*, 2003).

Landscape genetics' studies have been mainly focused on investigating landscape effects on gene flow, correlating the spatial heterogeneity and characteristics of landscape with estimates of genetic distance between individuals or populations (Cushman *et al.*, 2006; Balkenhol *et al.*, 2009). Research in this field has provided a connection between the theoretical study of gene flow (characterized only by geographical distances) and the study of genetic variability in heterogeneous and fragmented landscapes, providing estimates of functional connectivity (Manel & Holderegger, 2013). Another focus of landscape genetics studies is the identification of patterns of adaptive genetic diversity, allowing for the detection of which landscape factors influence the process of adaptation (Schoville *et al.*, 2012). This has led to the appearance of the branch of landscape genomics, which uses genome scans with large numbers of genotyped molecular markers, to investigate adaptive patterns of species driven by environmental and landscape factors (Luikart *et al.*, 2003; Razgour *et al.*, 2017).

Due to its novel outputs, landscape genetics analyses provide new promising information for conservation management (Segelbacher *et al.*, 2010; Sommer *et al.*, 2013). Several studies have been focusing on species-specific management issues, aiming to better inform on species' dispersal barriers or corridors, for more efficient conservation practices (Keller *et al.*, 2015). Nevertheless, even though landscape genetics methods can provide relevant information for conservation planning, the practical applications of these studies have seldom been used by practitioners or applied in conservation decision-making processes (Keller *et al.*, 2015). It would also seem that the outputs of landscape genetics' studies on the inference of connectivity between individuals or populations, frequently lacks a quantitative approach to evaluate which areas of species habitats or population patches should be prioritized for conservation (Storfer *et al.*, 2007; Galpern *et al.*, 2012). Landscape genetics' identification of putative gene flow and dispersal routes is currently not enough to present applicability in conservation management, as connectivity must also be considered in planning for species' and population's viability. Solely consideration of genetic measures may lead to inaccurate estimations of connectivity (Samarasin *et al.*, 2017). As such, the integration of landscape genetics with connectivity methodologies is of great interest, as it would provide quantitative information on priority areas, allowing the design of spatially explicit conservation measures.

1.4 CONNECTIVITY FOR CONSERVATION

Connectivity has been recently deemed of utmost relevance in conservation management, as it portrays the fragmentation of species populations and habitats, allowing for the evaluation of which areas of species' distributions should be prioritized in conservation (Ament *et al.*, 2014). It can be defined as the movement of individuals and genes among resource patches, relating to dispersal across landscapes (Vasudev *et al.*, 2015). Connectivity is known to shape species' population structure and dynamics, distribution patterns and demographics, while also influencing gene flow, genetic variability and evolutionary processes. Restrictions in connectivity, and therefore gene flow, within species, may cause the loss of genetic variability leading to the isolation, and even extinction, of populations (Galpern *et al.*, 2012; Koen *et al.*, 2014). As such, the maintenance of connectivity is of extreme relevance in species' conservation (Saura *et al.*, 2011; Saura & de la Fuente, 2017), as it counters the adverse effects of habitat and landscape fragmentation, one of the main causes of global biodiversity loss (Tournant *et al.*, 2013).

As a consequence of its importance in biodiversity conservation, several methodologies and measures have been developed for connectivity analyses. Molecular tools may be used to estimate genetic connectivity, by using measures such as migration rate and gene flow, which quantify the level of movement among populations (Broquet & Petit, 2009; Dyer *et al.*, 2010). Also, in landscape genetics' studies, there's a general usage of indirect measures of gene movement, such as genetic distance or genetic differentiation, to infer connectivity (Epps & Keyghobadi, 2015). In ecology, there are numerous connectivity measures, such as structural (e.g. nearest neighbour distance, spatial pattern indices), potential (e.g. graph-theoretic measures, incidence function metapopulation models) and functional (e.g. observed emigration, immigration, dispersal rates) connectivity metrics (Calabrese & Fagan, 2004). These are based on the physical properties and structure of landscapes (e.g. number and size of patches, parcelization, land-use) and on the movement of individuals among patches, taking into account species' ecological patterns (e.g. number of individuals, reproduction rates, mortality during dispersal). However, the more accurate metrics, such as methods of direct observation of connectivity, tend to have higher data requirements and are only feasible to be applied in smaller scales (Calabrese & Fagan, 2004). In order to facilitate the applicability of connectivity metrics, several spatially-explicit methodologies and softwares have been developed, such as Circuitscape (McRae, 2006; McRae *et al.*, 2008), which uses algorithms from electronic circuit theory to predict movement patterns (e.g. dispersal, gene flow) among individuals or populations in heterogeneous landscapes. Circuitscape is frequently used in

combination with landscape genetics to infer on species' probable gene flow and genetic dispersal routes (e.g. Polato *et al.*, 2011; Epps *et al.*, 2013; Dupas *et al.*, 2014; Marrotte *et al.*, 2014; Razgour *et al.*, 2014) and is also applied in conservation studies and planning (e.g. McRae *et al.*, 2008; Roever *et al.*, 2013; Brodie *et al.*, 2015; Lechner *et al.*, 2015; Vasudev & Fletcher, 2015). Another recently developed software, Conefor (Saura & Torné, 2009), has shown significant applicability in conservation management studies (e.g. Saura & Pascual-Hortal, 2007; Saura *et al.*, 2011; Clauzel *et al.*, 2015; Maiorano *et al.*, 2015; Rappaport *et al.*, 2015; Wang *et al.*, 2016). This software quantifies, through connectivity indices, the importance of habitat patches and links for the maintenance of landscape connectivity, while also identifying priority areas for conservation. However, despite presenting promising outputs for spatial conservation prioritization, this methodology has rarely been integrated with molecular analyses. By combining landscape genetics' methodologies with connectivity analyses, particularly with spatial identification of areas at risk of fragmentation, can present as a powerful tool for conservation management.

1.4 PURPOSE OF THIS RESEARCH

The current biodiversity crisis calls for the need for the development of novel methodologies to aid in conservation management. Recent advances in ecological and molecular techniques have allowed for a better understanding of species biology, namely their distribution patterns, ecological preferences and genetic structure, providing relevant insights for the evaluation of species' conservation status, more efficient conservation planning and determination of possible threats. Moreover, as new and cryptic species are being discovered, it becomes of utmost conservation priority the rapid characterisation of their ecological and genetic features. This study aims to combine molecular analyses with predictive modelling to provide relevant ecological and genetic insights on newly discovered cryptic species of bats, presenting relevant outcomes for conservation management.

Bats have experienced a global decline in populations over the last century, with almost 16% of species within this group being highly threatened or extinct (IUCN, 2017). Despite their biological diversity and ecological significance (Whittaker & Jones, 1994; Altringham, 1996; Cleveland *et al.*, 2006; Kalka *et al.*, 2008; Boyles *et al.*, 2011), bats face numerous threats, namely destruction of foraging habitat and roosting sites, roost disturbance and pesticide use (Stebbings, 1988; Hutson & Mickleburgh, 2001; Jones *et al.*, 2009). More recently, fatalities recorded at wind farms and roads have increased the impacts on bat populations, along with the emerging disease of white nose syndrome (Kunz *et al.*, 2007; Arnett *et al.*, 2008;

Rodrigues *et al.*, 2008; Baerwald *et al.*, 2009; Blehert *et al.*, 2009; Kerth & Melber, 2009; Rydell *et al.*, 2010; Lesiński *et al.*, 2011; Langwig *et al.*, 2012). Bats present high vulnerability to negative impacts in populations, mainly due to their slow population growth, late sexual maturity and dependence on specific roosting and foraging habitats (Jones *et al.*, 2003; Safi & Kerth, 2004; Jones *et al.*, 2009), translating in elevated extinction risks. Consequently, several conservation policies have been established in an attempt to reduce the threats to bat species worldwide, such as the recent "Year of the Bat (2011-2012)" campaign, created by the United Nations through the UNEP (United Nations Environmental Programme) Convention on Migratory Species (CMS), and The Agreement on the Conservation of Populations of European Bats (EUROBATS). Moreover, bats are protected by the Bern Convention on the Conservation of European Wildlife and Natural Habitats of 1982, the Bonn Convention on the Conservation of Migratory Species of Wild Animals of 1983, and the Habitats Directive of 1992 (Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora).

Due to the advent of molecular techniques, the discovery of new bat species and cryptic complexes have significantly increased bat diversity worldwide (Mayer & von Helversen, 2001; Ibáñez *et al.*, 2006; Mayer *et al.*, 2007; Sun *et al.*, 2009; Puechmaille *et al.*, 2014). Cryptic species can be defined as ecologically and/or genetically distinct species, usually mistakenly classified and hidden under one species name, which typically possess indistinguishable morphology (Jones, 1997). These species pose as a challenge to biodiversity conservation, as they not only may lead to the underestimation of species' diversity, but also present endangered taxa within the species complex, in need of a deeper understanding of species ecological and conservation requirements (Bickford *et al.*, 2006; Sattler *et al.*, 2007).

In particular, the Iberian Peninsula has presented substantial amounts of bat cryptic diversity (Mayer & von Helversen, 2001; Agirre-Mendi *et al.*, 2004; Juste *et al.*, 2004; Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Salicini *et al.*, 2013). This rich genetic diversity, is mainly due to Iberia's placement within the Mediterranean basin, one of the most important biodiversity hotspots (Myers *et al.*, 2000; Schipper *et al.*, 2008) and a major Pleistocene glacial refugium (Hewitt, 2000; Gómez & Lunt, 2007). Due to its location, however, it is predicted to be one of the areas in the world where climate change impacts are expected to be most severe, especially regarding the increase of aridity and drought episodes (Milly *et al.*, 2005; Dai, 2011). In this context, the Iberian Peninsula presents itself as the area of focus of the work developed in this thesis.

The Iberian Peninsula is located in south-western Europe with a total area of nearly 600 000 km^2. It is enclosed by the Mediterranean Sea in the south and east, and in the north and west by the Atlantic Ocean. It possesses strong heterogeneity in topography and climate, being characterised by two main biogeographical regions, Eurosiberian and Mediterranean (Gómez & Lunt, 2007; Sillero *et al.*, 2009; Romo & García-Barros, 2010). From a conservation perspective, both countries in Iberia undersign the majority of Environmental International agreements, with more than 20% of the land classified as protected areas (Araújo *et al.*, 2007).

For their ecological, molecular and conservation relevance, this work will be focusing on the bat cryptic species in the Iberian Peninsula, particularly on the recently described Iberian subspecies of the Brown long-eared bat, *Plecotus auritus begognae* de Paz, 1994. As a newly described taxon, it is of conservation concern to clarify its distribution, ecological requirements and population structure. Resembling its sibling, *Plecotus auritus*, *P. a. begognae* is a tree-dwelling bat, presenting a strong affinity to woodland and forested habitats (Entwistle *et al.*, 1996). They are relatively slow flyers (Norberg & Rayner, 1987) and, therefore, present limited dispersal capabilities (Burland *et al.*, 1998), translating in a higher risk of vulnerability with changes in habitat or environmental conditions, and fragmentation.

The proposed objectives of this thesis are:

1 – To unravel the distributions and environmental affinities of bat cryptic species in Iberia, by building habitat-suitability models, determining which ecological factors limit their distribution and establish possible contact zones between populations
2 – To clarify the evolutionary history of the Iberian long-eared bats, providing further evidence on the taxonomic status of *P. a. begognae*
3 – To determine *P. a. begognae*'s population structure and identify the connectivity structure between populations, unveiling possible barriers to gene flow and priority areas for conservation
4 – To predict *P. a. begognae*'s distribution in the future considering climate change scenarios and determining possible extinction risk by integrating barriers to gene flow.

1.5 THESIS OUTLINE

The initial chapters in this thesis aim at clarifying the genetic, environmental and distributional characteristics of the focus species, providing insights for field survey planning. Progression to the subsequent chapters was only achieved with the data obtained as a result of the studies conducted in the first chapters. The last chapters focus on determining the environmental drivers shaping species' genetic structure, while also revealing the species' distributional areas most susceptible to extinction or fragmentation, in the present and in future climate change scenarios.

Chapter two investigates the environmental and ecological characteristics shaping the distributions of cryptic bats in the Iberian Peninsula, considering their ecological interactions and biogeographical patterns. Three cryptic bat species complexes were the focus of this study, *Plecotus auritus auritus*/*auritus begognae*, *Myotis mystacinus*/*alcathoe* and *Eptesicus serotinus*/*isabellinus*. Calculated SDMs for each species were overlapped to inform on each complexes' contact zones and niche analyses were performed to infer niche overlap and breadth. Results revealed species' distributional ranges, ecological niches and biogeographical affinities, while allowing for the inference of new occurrence areas. Field campaigns were then successfully carried out in these areas, in order to fill sampling gaps.

Due to the lack of significant knowledge on the taxonomic status and phylogenetic structure of *P. a. begognae*, chapter three aimed at clarifying the evolutionary history of the Iberian species of the genus *Plecotus*. Mitochondrial, nuclear and microsatellite markers were used to shed a light on the *Plecotus* spp. lineages present in the Iberian Peninsula. Phylogenetic analyses were combined with microsatellite data to better infer on the lineage's evolutionary relationships and differentiations. All phylogenetic reconstructions supported the separation of the *P. austriacus* and *P. macrobullaris* groups, while the lineages *P. a. auritus* and *P. a. begognae* were only differentiated at the mitochondrial level. Microsatellite analyses, however, showed a clear separation of all the *Plecotus* lineages (*austriacus*, *macrobullaris*, *a. auritus* and *a. begognae*), while demonstrating low levels of gene flow occurring between *P. a. auritus* and *P. a. begognae*. These results allowed to infer on the possible past isolation of Iberian populations of *P. a. auritus* from their European counterpart, leading to the differentiation of an Iberian lineage, *P. a. begognae*. Moreover, these outputs demonstrate the importance of *P. a. begognae* as an evolutionary significant unit, relevant for conservation management, and support the subspecies status of *P. a. begognae*.

Newly gathered records of previously unknown distribution areas of *P. a. begognae* were joined with samples collected by specialists throughout a decade in the Iberian Peninsula, to form a highly representative dataset of the species range. All records were identified through microsatellite analyses, and this dataset was then employed in chapters four and five.

Chapter four presents a multi-disciplinary approach to study the environmental drivers affecting the population structure and connectivity patterns of *P. a. begognae* in the Iberian Peninsula. Molecular analyses were combined with distribution modelling, landscape genetics and connectivity methodologies to infer on what environmental features shaped species gene flow and dispersal patterns within the study area, and identify the possible at-risk areas within the species distribution. Even though molecular analyses showed no population structure for *P. a. begognae*, landscape genetics results managed to demonstrate that gene flow is highly dependent of forested and steep sloped areas. Connectivity analyses revealed that areas in the north-west and centre-east of the Iberian Peninsula are highly susceptible to isolation, since their connectivity to the species' main distributional core is occurring over small forested areas. These results also allowed us to infer that the large extents of forests in the northern and central regions of Iberia seem to be preventing the fragmentation of the populations of *P. a. begognae*. A significant conservation output was obtained, since the combination of landscape genetics analyses with connectivity methodologies presented spatially explicit results that allowed for the identification of the areas sensitive to genetic isolation.

Finally, chapter five aimed at applying the previously developed multi-disciplinary approach to infer on the impacts of climate change on the populations of *P. a. begognae* in the Iberian Peninsula. Climatic modelling was combined with landscape genetics analyses, to assess how this species will shift its range and which are the most probable dispersal routes. This framework was applied to climate change scenarios for 2050 and 2070. Results indicated that most of the species distributional range will suffer a severe contraction, with several large suitable areas disappearing entirely. Most of the range shift pathways seem to be located along the eastern and northern coasts of the Iberian Peninsula, as are the areas that seem to remain suitable throughout the decades of climate change scenarios. However, central and western located movement routes showed to be of utmost relevance, since distribution patches located in those areas will ultimately disappear, and, as such, connection routes to suitable areas need to be maintained. By using this framework, we were able to identify *P. a. begognae*'s pathways in potential future range shifts, as well as inform on conservation management, by determining the priority areas for increased landscape connectivity and movement facilitation in climate change scenarios.

CHAPTER 2

ECOLOGY AND DISTRIBUTION OF CRYPTIC BATS IN THE IBERIAN PENINSULA

PAPER I - INFLUENCES OF ECOLOGY AND BIOGEOGRAPHY ON SHAPING THE DISTRIBUTIONS OF CRYPTIC SPECIES: THREE BAT TALES IN IBERIA

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ABSTRACT

To determine what shapes the distributions of cryptic species, we aimed to unravel ecological niches and geographical distributions of three cryptic bat species complexes in Iberia, *Plecotus auritus/begognae*, *Myotis mystacinus/alcathoe* and *Eptesicus serotinus/isabellinus* (with 44, 69, 66, 27, 121 and 216 records, respectively), considering ecological interactions and biogeographical patterns. Species distribution models (SDMs) were built using a presence-only technique (Maxent), incorporating genetically identified species records with environmental variables (climate, habitat, topography). The most relevant variables for each species' distribution and respective response curves were then determined. SDMs for each species were overlapped to assess the contact zones within each complex. Niche analyses were performed using niche metrics and spatial principal component analyses to study niche overlap and breadth. The *Plecotus* complex showed a parapatric distribution, although having similar biogeographical affinities (Eurosiberian), possibly explained by competitive exclusion. The *Myotis* complex also showed Eurosiberian affinities, with high overlap between niches and distribution, suggesting resource partitioning between species. Finally, *E. serotinus* was associated with Eurosiberian areas, while *E. isabellinus* occurred in Mediterranean areas, suggesting possible competition in their restricted contact zone. This study highlights the relevance of considering potential ecological interactions between similarly ecological species when assessing species distributions.

INTRODUCTION

Recent concerns regarding biodiversity loss call for a deeper understanding of current species distributions patterns and how those patterns are shaped. This knowledge is highly relevant when accounting for species conservation planning and fundamental in ecological and evolutionary studies (Jetz *et al.*, 2007). Cryptic species, defined as ecologically and/or genetically distinct species with very similar morphology (Jones, 1997), pose a challenge to understanding species distributions as they bring the need to redefine what was once thought to be a single species' distribution and environmental requirements. Moreover, cryptic species may lead to biodiversity underestimates and may comprise threatened taxa within the cryptic complex that consequently require new conservation statuses (Bickford *et al.*, 2006; Sattler *et al.*, 2007).

Due to increasingly rapid DNA sequencing and the advances in molecular phylogenetic methods over the past decades, many cryptic species have recently been identified (e.g. (Chadès *et al.*, 2008; Kaliontzopoulou *et al.*, 2011; Boratynski *et al.*, 2012). Indeed, one of the most diverse mammal groups in Europe – bats – has significantly increased in species number (Mayer & von Helversen, 2001; Ibáñez *et al.*, 2006). In this context, the Iberian Peninsula possesses rich genetic diversity, mainly due to it being a major glacial refugium for several species (Hewitt, 2000; Gómez & Lunt, 2006; Razgour *et al.*, 2013), making the Mediterranean basin an important biodiversity hotspot (Myers *et al.*, 2000). Recent evidence suggests that 20% of Iberian bat species, which comprise nearly half of Iberia's mammal species (Mitchell-Jones *et al.*, 1999), harbour complexes of cryptic species (Juste *et al.*, 2004; Ibáñez *et al.*, 2006). There are six recently discovered cryptic bat species complexes in the Iberian Peninsula: *Plecotus auritus* (Linnaeus, 1758)/*begognae* (de Paz, 1994)/*macrobullaris* (Kuzyakin, 1965), *Myotis mystacinus* (Kuhl, 1817)/*alcathoe* (Helversen & Heller, 2001), *Eptesicus serotinus* (Schreber, 1774)/*isabellinus* (Temminck, 1840), *Myotis escalerai* (Cabrera, 1904)/ sp.1, *Hypsugo savii*'s (Bonaparte, 1837) complex and *Pipistrellus kuhlii*'s (Kuhl, 1817) complex (Agirre-Mendi *et al.*, 2004; Ibáñez *et al.*, 2006). Despite the discovery of genetic discontinuities in these complexes, only *P. auritus/begognae/macrobullaris*, *M. mystacinus/alcathoe*, *E. serotinus/isabellinus* and *M. escalerai*/sp.1 exhibit high nucleotide divergence between the lineages (mtDNA distance over 8%) (Ibáñez *et al.*, 2006), indicating that these taxa may have experienced isolation during glacial periods (Pavan *et al.*, 2011). The *M. escalerai*/sp.1 complex has already been the focus of other studies (Salicini *et al.*, 2013) and we had very few data for *P. macrobullaris* so this species will not be considered in this study. Although these studies have provided genetic information, there is currently no information about these species' distributions or their ecological requirements in Iberia, or how these species are able to coexist.

The geographical distribution of a species is delineated by its ecological properties, namely the environmental conditions favourable for its occurrence, species interactions and dispersal capacity (Peterson, 2011). Environmental conditions (such as climate, habitat and availability of resources) will shape species distributions according to the geographical space where they meet species requirements for survival. Interactions with other species, either negative (competition, predation) or positive (facilitation), will further limit species distributions. All these factors will define the currently occupied distribution of a species, reflecting the accessible geographical space in which both biotic and abiotic conditions allow species existence (Peterson, 2011).

The use of species distribution models (SDMs) minimizes this caveat, by determining species potential distributions along with their environmental requirements. SDMs have been used effectively in several studies, aiding in the unravelling of the distributions of rare and cryptic species (Pearson *et al.*, 2007; Pineda & Lobo, 2009; Williams *et al.*, 2009; Rebelo *et al.*, 2010; Rutishauser *et al.*, 2012; Bosso *et al.*, 2013). The data used in SDMs should enclose all the conditions explored by the studied species (Wisz *et al.*, 2008) although frequently disregard biotic interactions. However, it is recognized that interactions such as competition, predation or parasitism may restrict the environments in which a species may live, forcing it to occupy a narrower set of conditions (Hutchinson, 1957; Soberón & Peterson, 2005). Competition can have relevant ecological effects on the niches of species and, if these interactions are strong and pervasive enough, they may produce an evolutionary response in species' populations, for example, by competitive exclusion (Anderson *et al.*, 2002). However, competition can lead to a specialization of the resources explored by each species, allowing stable coexistence through time by species-specific differentiation in resource utilization (Hutchinson, 1978; Wang *et al.*, 2005). Trophic resource partitioning is known to occur in several cryptic bat species in Europe, including *Myotis myotis* and M. blythii (Arlettaz *et al.*, 1997; Furman *et al.*, 2013), and habitat resource partitioning occurs in *Pipistrellus pipistrellus* and *P. pygmaeus* (Nicholls & Racey, 2006). These species coexist in sympatry, although they exploit different resources, such as prey, in the former case, or microhabitats, in the latter case.

The main purpose of this paper was to understand what shapes the distributions of cryptic species of bats in Iberia, and whether species share the same ecological background, thus developing hypotheses for current ecological interactions between cryptic species. Focusing on the cryptic complexes *P. auritus/begognae*, *M. mystacinus/alcathoe* and *E. serotinus/isabellinus* as case studies, the main questions addressed in this study were: (1) What is the spatial pattern of bat cryptic diversity in Iberia? (2) Which ecological factors limit those patterns? (3) Do cryptic species share the same ecological conditions, i.e. are limiting factors similar? (4) Do biogeographical affinities shape these species' distributions and contact zones in Iberia?

MATERIALS AND METHODS

Study Area

The study area was the Iberian Peninsula and the Balearic Islands, located in Europe's south-western extremity (Fig. 1.1a). It covers nearly 600 000 km2 and it is bordered to the south and east by the Mediterranean Sea and to the north and west by the Atlantic Ocean, being divided from the rest of Europe by the Pyrenees mountain range in the north-east. It has a very heterogeneous topography and is roughly characterized by two main biogeographical regions: Eurosiberian and Mediterranean (Sillero *et al.*, 2009; Romo & García-Barros, 2010).

Sample Collection

Distribution data for *Plecotus auritus/begognae*, *Myotis mystacinus/alcathoe* and *Eptesicus serotinus/isabellinus* were obtained from mist netting and roost trapping sessions in the Iberian Peninsula over the last decade (Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Rebelo *et al.*, 2012; Salicini *et al.*, 2013). For each specimen, a tissue sample was collected in the field, through a small biopsy punch in the wing membrane. Afterwards, species identification for all records was validated by molecular analyses, following the procedure described by (Ibáñez *et al.*, 2006) (see Appendix S1, Supporting information I). Using phylogenetic analyses, we also compared sequences from samples collected for this study with those published in the above-mentioned studies (Appendix S2, Supporting information I).

Prior to model calculations, spatial autocorrelation analyses were performed until data for each species achieved independence. For this, we used average nearest-neighbour analyses to remove clusters in the species' data. Consequently, for modelling there were 121 presence records for *E. serotinus*, 216 for *E. isabellinus*, 44 for *P. auritus*, 69 for *P. begognae*, 66 for *M. mystacinus* and *27 for M. alcathoe*.

Ecogeographical Variables

An initial set of 45 ecogeographical variables (EGVs) were chosen as predictors (see Table S3, Appendix S3, Supporting information I). The chosen set of EGVs aimed to represent the environmental predictors related to bat occurrence (Ulrich *et al.*, 2007). Climate conditions are related to bat physiology, energy demands and water availability (Racey *et al.*, 1987; Webb *et*

al., 1995; Adams & Hayes, 2008; Frick *et al.*, 2010). Land cover, and distances to different habitat classes and to slopes are associated with potential foraging areas and roosts for tree- and crevice-dwelling bats (Russo & Jones, 2003; Rainho & Palmeirim, 2011). Climatic variables were obtained from WorldClim (http://www.worldclim.org), topographical variables were obtained from the digital elevation data of the NASA Shuttle Radar Topography Mission (http://srtm.csi.cgiar.org) and habitat variables were obtained from the Globcover project (http://postel.obs-mip.fr/?GLOBCOVER-Project) and from 'Mapa Forestal de España' (Ruiz del Castillo *et al.*, 2006) for the eucalyptus data. All variables had a resolution of 300 × 300 m and respective calculations were made in ArcGIS 10.0 (ESRI, 2010).

Species Distribution Modelling

SDMs were built using the maximum entropy modelling technique, Maxent version 3.3.3k (Phillips *et al.*, 2006). Due to its reliability when using presence-only data, this technique has proven to outperform other modelling methods even with limited datasets (Hernandez *et al.*, 2006; Wisz *et al.*, 2008; Elith *et al.*, 2010; Rebelo & Jones, 2010). Bats are known for their elusive and nocturnal behaviour (Ahlén & Baagoe, 1999), thus presenting limited detectability and identification in flight. The use of presence-only data in our study aims to overcome the issue of 'false absences', referring to situations when a species was not detected although it was present (Elith *et al.*, 2010).

In a first approach, species presence records, as the dependent variables, and the selected EGVs, as the independent variables, were imported into Maxent and run in auto features with a regularization multiplier of 2. The regularization multiplier was selected after model selection tests calculated trough ENM Tools 1.3 (Warren *et al.*, 2010) (http://enmtools.blogspot.com) and chosen according to its Akaike information criterion corrected for small sample sizes (AICc) value. Afterwards, 20 model replicates were run using cross-validation, in which the whole presence data set is randomly split into equal-sized partitions. The area under the curve (AUC) of the receiver operating characteristics (ROCs) plot was taken as a measure of the overall fit of the models (Fielding & Bell, 1997). The AUC ranges from 0, complete randomness, to 1, perfect discrimination (Phillips *et al.*, 2006).
To decrease the number of variables for the final distribution models, we first eliminated the highly correlated variables by first calculating a correlation matrix and then selecting the pairs of variables with correlations above 0.80 (Elith *et al.*, 2010). From these correlated groups of variables, only the most relevant variables for all species were considered, by using the percentage contribution values and the jackknife values of regularized training gain (a

measure of likelihood between species presence data and the variable) obtained in Maxent for each species (Elith *et al.*, 2011).

A selected set of 15 variables (Table 1.1) was then used to build the final models, which we ran using the same settings as the initial models. Using these results, variable contribution plots and univariate response curves for the four most important variables for each species were built in Statistica© software.

Table 1.1 - Set of variables used in the final species distribution models

Type	Variable		Code
Climatic	Annual Mean Temperature (ºC)		ib_bio1
	Mean Diurnal Range (ºC)		ib_bio2
	Mean Temperature of Warmest Quarter (ºC)		ib_bio10
	Mean Temperature of Coldest Quarter (ºC)		ib_bio11
	Precipitation of Wettest Quarter (mm)		ib_bio16
	Precipitation of Driest Quarter (mm)		ib_bio17
Topographical	Altitude (m)		ib_dem
	Maximum slope (º)		ib_slopemax
	Distance to slope >20° (m)		ib_di_sl20
	Distance to maximum slope >20°(m)		ib_di_slmax20
Habitat		Land cover:	
		Agriculture 1	
		Orchards 2	
		Forested agriculture 3	
		Forest 4	
		Coniferous 5	ib_land
		Shrubs 6	
		Bare 7	
		Urban 8	
		Water 9	
		Eucalyptus plantations 10	
	Distance to forests (m)		ib_di_forest
	Distance to agriculture (m)		ib_di_agric
	Distance to water bodies (m)		ib_di_water
	Distance to eucalyptus plantations (m)		ib_di_eucal

The SDMs built were then imported into ArcGIS 10.0 and reclassified into presence–absence using the maximum training sensitivity plus specificity logistic threshold value (Liu *et al.*, 2013). The reclassified models were then overlapped to define the possible contact zones between the species within each cryptic complex.

Niche Analyses

Using the final SDMs calculated we proceeded to analyse the ecological niches of each species. Niche overlap (Warren *et al.*, 2008) and niche breadth (Nakazato *et al.*, 2010) statistics for each species were calculated using ENMTools 1.3. Niche overlap analyses were applied within each cryptic complex and considered the values of three indexes, Schoener's D (Schoener, 1968), the I statistic (Warren *et al.*, 2008) and relative rank (Warren & Seifert, 2011). Also, Levin's index (Levins, 1968), to determine niche breadth, was calculated for each species. Spatial principal components analysis (sPCA) was used to visualize the ecological niches of the different species. Initially, we extracted the values of each EGV for each grid cell of our study area, then, using these values, a PCA was calculated in R version 2.15.3 (R Core Team, 2012). The resulting values of the PCA components were then imported into ArcGIS 10.0 and the values of each species presence records were plotted for each component. We calculated minimum convex polygons to delimit each species environmental space from the samples included in this study (hereafter termed the realized niche) and measured their individual areas and the area of overlap between species' niches.

RESULTS

Important ecogeographical variables for species distributions

The variables that contributed the most to the distribution models built differed among species. Graphs of variable contributions and respective response curves can be found in Figures S3.1–S3.12 in Appendix S3, Supporting information I. When analysing the response curves for these variables we can see that both *P. auritus* and *P. begognae* are more likely to occur at low temperatures and in areas with high precipitation. *Plecotus begognae* also favoured the presence of steep slopes. Low temperatures and proximity to steep slopes seem to favour the occurrence of *M. mystacinus*. The response curves of *M. alcathoe* showed that this species' occurrence is more likely in areas with high precipitation and in close proximity to forests and

to steep slopes. In the case of *E. serotinus*, occurrence is most favoured at high precipitation and in close proximity to forests and steep slopes. Finally, *E. isabellinus* seems to have a higher probability of occurrence in areas with moderate temperatures, low precipitation and within and in the vicinity of eucalyptus plantations.

Species distributions and contact zones

The SDMs built exhibited ROC curves with high average AUCs, with all species presenting similar values, these being 0.98 ± 0.016, 0.91 ± 0.025, 0.91 ± 0.054, 0.96 ± 0.015, 0.86 ± 0.065 and 0.92 ± 0.034, for *P. auritus*, *P. begognae*, *M. mystacinus*, *M. alcathoe*, *E. serotinus* and *E. isabellinus*, respectively. In the case of the *P. auritus/begognae* complex (Fig. 1.1b), the models predicted the distribution of P. auritus to be restricted to the Pyrenees area and *P. begognae* to occur mostly through northern Iberia and the Balearic Islands with a patchy distribution in the central region mountains. Due to these focused distributions, the contact zone of these two species seems to be delimited by the low elevations of the Pyrenees. When considering the model's results for the *M. mystacinus/alcathoe* complex (Fig. 1.1c), it seems that both species have a northern distribution in Iberia, with *M. mystacinus*' distribution extending further south than *M. alcathoe*'s. Also, *M. alcathoe*'s distribution is mostly contained within the wider distribution of *M. mystacinus*. Finally, the models demonstrated a high degree of separation for *E. serotinus/isabellinus* (Fig. 1.1d) in Iberia. Although somewhat patchy, the distribution of *E. serotinus* was mainly concentrated in the north of Iberia and in the Balearic Islands. The distribution of *E. isabellinus* is focused in the south of Iberia, and it is not predicted to occur in the Balearic Islands. Accordingly, the contact zone between these two species' distributions appears to be in the central regions of Iberia.

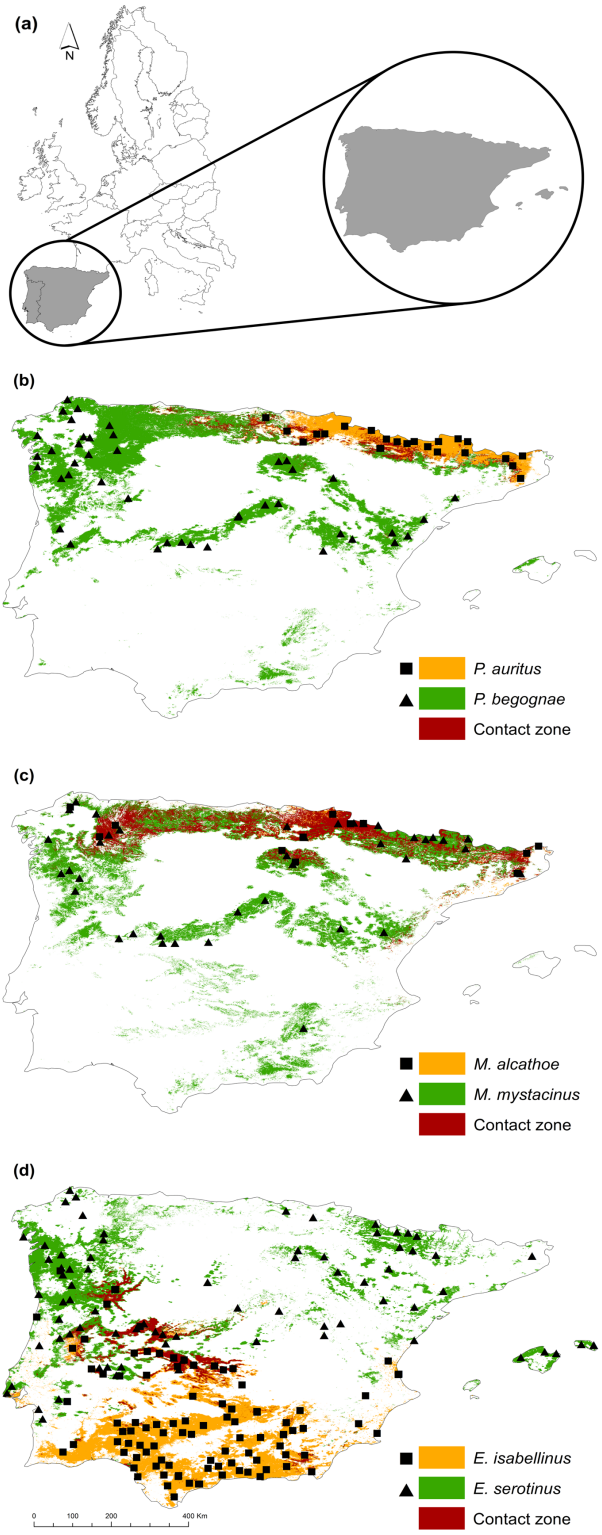

Figure 1.1 - The study area in the European context (a). Potential distribution and presence records of the (b) *Plecotus auritus/begognae* complex, (c) *Myotis mystacinus/ alcathoe* complex and (d) *Eptesicus serotinus/isabellinus* complex. Each symbol may correspond to more than one location.

Niche Analyses

For the analyses of niche overlap and niche breadth using the distribution models calculated for each species, we present the overlap statistics for each cryptic complex (Table 1.2) and the niche breath values for each species individually (Table 1.3). Also, the sPCA built allowed us to determine the overlap between the occupied species' environmental niches in Iberia (Table 1.4).

Table 1.2 - Niche overlap statistics

Schoener's D		***I* statistic**		**Relative rank**	
	P. begognae		*P. begognae*		*P. begognae*
P. auritus	0.11	*P. auritus*	0.26	*P. auritus*	0.77
	M. mystacinus		*M. mystacinus*		*M. mystacinus*
M. alcathoe	0.48	*M. alcathoe*	0.77	*M. alcathoe*	0.82
	E. serotinus		*E. serotinus*		*E. serotinus*
E. isabellinus	0.29	*E. isabellinus*	0.54	*E. isabellinus*	0.50

Table 1.3 - Niche breath (Levin's Index)

	Inverse concentration	**Uncertainty**
P. auritus	0.01	0.77
P. begognae	0.12	0.90
M. mystacinus	0.12	0.90
M. alcathoe	0.05	0.86
E. serotinus	0.22	0.95
E. isabellinus	0.10	0.91

Table 1.4 - Overlap values of sPCA analyses

	Realized niche		
Species	**Area**	**Overlap (%)**	**Area in Iberia (%)**
P. auritus	8.54	25.64	8.45
P. begognae	11.85	18.48	11.72
M. mystacinus	18.06	22.81	17.87
M. alcathoe	4.12	100.00	4.08
E. serotinus	30.07	35.42	29.75
E. isabellinus	20.86	51.05	20.64

The sPCA obtained for the *P. auritus/begognae* complex (Fig. 1.2a) shows that both species have different and somewhat restricted niches within Iberia. *Plecotus begognae*'s niche (Area = 11.85) is slightly broader than that of *P. auritus* (Area = 8.54) and the overlap between both species' niches is similar (25.64% for *P. auritus* and 18.48% for *P. begognae*), with *P. auritus* having a slightly larger amount of its niche area within *P. begognae*'s niche (Table 1.4). In the case of the *M. mystacinus/alcathoe* complex (Fig. 1.2b), both species seem to occupy similar conditions in Iberia, although *M. alcathoe*'s niche area (Area = 4.12) is much smaller than *M. mystacinus*' (Area = 18.06). Moreover, *M. alcathoe*'s niche is completely within that predicted for *M. mystacinus*, while only 22.81% of the niche area of *M. mystacinus* is occupied by *M. alcathoe*'s niche. Lastly, in the *E. serotinus/isabellinus* complex (Fig. 1.2c), both species occur in a relatively large range of conditions in Iberia. *Eptesicus isabellinus* has half of its niche overlapping with *E. serotinus*, whilst *E. serotinus* only has 35.42% of its niche within that predicted for E. *isabellinus*.

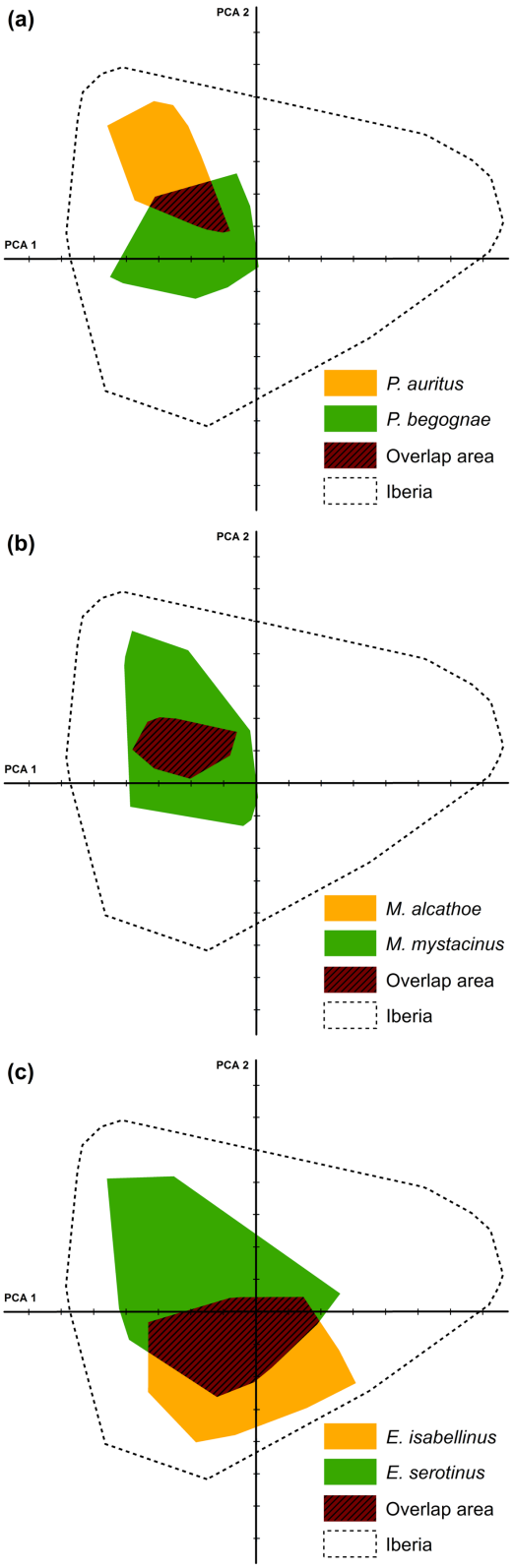

Figure 1.2 - Spatial principal component analyses of the ecological niches of (a) *Plecotus auritus/begognae* complex, (b) *Myotis mystacinus/alcathoe* complex and (c) *Eptesicus serotinus/isabellinus* in Iberia. Filled polygons represent the realized niche and the dotted line represents the environmental space available in Iberia.

When analysing the sPCA, we were able to distinguish two main biogeographical patterns with two strata, Eurosiberian and Mediterranean (Fig. 1.3). As for the biogeographical patterns of the contact zones between each complex, we can see contact zones for *P. auritus/begognae* (Fig. 1.3a) and *M. mystacinus/alcathoe* (Fig. 1.3b) overlapping in relatively the same biogeographical area located in the north of the Iberian Peninsula. As *M. mystacinus/alcathoe*'s contact zone is broader, it occurs to a small extent in central Iberia, but mainly follows the same biogeographical pattern, i.e. present in the north, avoiding the biogeographical strata of the south. As for the *E. serotinus/isabellinus* complex (Fig. 1.3c), the pattern is less clear. The contact zone between these species seems to be mainly located in transition areas between the two main biogeographical strata, but is mainly situated in the central–western area of Iberia.

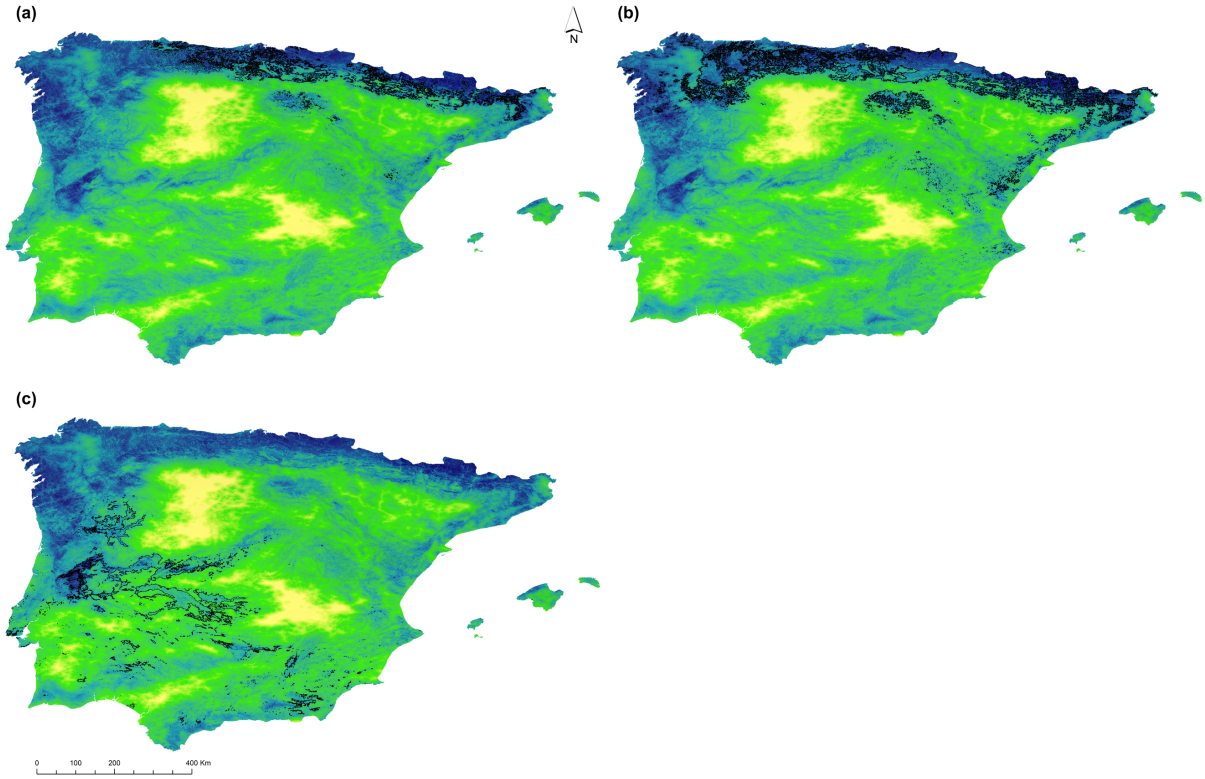

Figure 1.3 - Spatial principal component analysis with the two main biogeographical strata identified for Iberia. The blue areas (darker tones) represent Eurosiberian areas and the green to yellowish areas (lighter tones) Mediterranean areas. The contact zones of (a) the *Plecotus auritus/begognae* complex, (b) *Myotis mystacinus/alcathoe* complex and (c) *Eptesicus serotinus/isabellinus* complex are delineated in black.

DISCUSSION

This study reveals the potential distributions of newly discovered cryptic complexes of bats in the Iberian Peninsula and also, by analysing their niches individually and within complexes, aids in the understanding of these species' ecological requirements and how they may be affecting each other's distributions. Different distribution patterns were observed within and between complexes: most species were concentrated in the north of Iberia (*P. auritus*, *P. begognae*, *M. mystacinus*, *M. alcathoe* and *E. serotinus*), and one species occupied southern Iberia (*E. isabellinus*). These distribution patterns clearly associate with the known biogeographical affinities of each species (Horáček *et al.*, 2000), where the majority have a clear Eurosiberian/Atlantic distribution and only *E. isabellinus* shows an association with Mediterranean areas. When analysing species' ecological niches, we observed that *P. auritus* and *M. alcathoe* had considerably narrower niches when compared with the other species, as supported by studies that reveal these species as specialists (Helversen *et al.*, 2001; Ashrafi *et al.*, 2011). *Eptesicus serotinus* had the broadest niche, which was expected as it is known to be a generalist species (Catto *et al.*, 1996). Strong niche dynamics occur within complexes, resulting from either an established equilibrium or other ecological interactions that occur (e.g. competition) and realized niches in Iberia may still be adjusting. Historical factors should also be considered. For example, it is possible that some European lineages (such as *P. auritus*) have recently arrived in Iberia and species distribution limits may still be under definition processes. In fact, each of the species complexes focused on in this study seems to have a unique story to tell.

The *Plecotus* Tale

Both species distributions in the *Plecotus* complex seem to be highly related to climatic variables and to the presence of slopes. In fact, when analysing this complex's distribution patterns, *P. auritus* seems to mainly occur in the mountainous areas of the Pyrenees, while *P. begognae* occupies the rest of northern Iberia, notably excluding the Pyrenees. *Plecotus begognae*, a recently discovered lineage in the Iberian Peninsula, had a relatively unknown distribution, ecological requirements and biogeographical affinity. However, (de Paz, 1994) found this species in the central mountainous regions of Iberia, south of the Cantabrian Mountains and south of the Pyrenees, later confirmed by (Ibáñez *et al.*, 2006). Although both *Plecotus* species occupy the northern areas of Iberia and appear to have similar biogeographical affinities, their distributions have relatively little overlap restricted to a few areas located in north-eastern Iberia (south of the Pyrenees), reflected also in a small niche

overlap. This complex's parapatric distribution could be due to resource competition, leading to competitive exclusion. (Rutishauser *et al.*, 2012) found parapatric distributions in two *Plecotus* species in Switzerland (*P. austriacus* and *P. macrobullaris*) probably resulting from competitive exclusion, supported by strong similarities in their echolocation signals, flight behaviour (Dietrich *et al.*, 2006) and diet (Ashrafi *et al.*, 2011). Other bat species have been known to have parapatric distributions due to competitive exclusion; for example, *Vespertilio murinus* is excluded by *Eptesicus serotinus* on some Danish islands, presumably as a consequence of competition (Baagøe, 1986) or *P. pipistrellus* and *P. kuhlii* in southern Switzerland, where *P. kuhlii* seems to restrict *P. pipistrellus* to the uplands as a result of competitive pressure (Haffner & Stutz, 1985).

However, coexistence also occurs in *Plecotus* species; trophic niche partitioning exists between *P. auritus* and *P. macrobullaris*, and *P. auritus* and *P. austriacus* coexist probably by niche partitioning in Central Europe and the Alps (Ashrafi *et al.*, 2011). Therefore, only through small-scale studies (radiotracking, diet analyses, etc.) will it be possible to assess the interactions between *P. auritus* and *P. begognae*, and whether competition can potentially occur.

The *Myotis* Tale

Myotis mystacinus and *M. alcathoe* are restricted to the north of Iberia, with *M. mystacinus* showing a relatively wider distribution than *M. alcathoe*. Accordingly, both species occur in the Eurosiberian part of Iberia, mainly in mountainous regions (Agirre-Mendi *et al.*, 2004), which explains the relevance of climatic and topographic variables in explaining the distributions of these species. Although these species have similar distributions to the *Plecotus* complex, the overlap between them is rather different. *Myotis alcathoe*'s distribution is almost completely contained within the distribution of *M. mystacinus*, and its entire realized niche is within that of *M. mystacinus*. It appears that these species' distributions are sympatric and some sort of niche equilibrium is occurring between them. We suggest that stable coexistence may occur between these species, with a likely species-specific differentiation in resource utilization. Indeed, it is thought that *M. alcathoe* is sympatric with *M. mystacinus*, exploring mainly stands of deciduous trees near bodies of water (Helversen *et al.*, 2001), explaining the relevance of distance to forests in this species' distribution models. However, *M. alcathoe* differs from the other European *Myotis* species due to its restricted habitat requirements (Lučan *et al.*, 2009), hence suggesting that *M. alcathoe* and *M. mystacinus*, although with a highly sympatric distribution, are able to coexist, most likely by exploring different micro-habitats through

resource partitioning. In fact, (Helversen *et al.*, 2001) found that, in Greece, *M. mystacinus* appears to occur in forested marginal habitats, generally hunting near large bodies of water, whereas *M. alcathoe* is found in dense woodland, hunting along small streams. Such resource partitioning is known to occur in other sympatric species of the same genus, such as between *M. myotis* and *M. blythii* (Arlettaz *et al.*, 1997) and between *M. bechsteinii* and *M. naterreri* (Siemers & Swift, 2006), highlighting the existence of species- specific micro-habitat preferences.

The *Eptesicus* Tale

The *Eptesicus* complex is distinct from the patterns seen in the previous two complexes. Both *Eptesicus* species have distinct distributions in Iberia, with *E. serotinus* mainly in the north and *E. isabellinus* in the south. *Eptesicus serotinus* is known to occur in temperate climates (Rebelo *et al.*, 2010), and (Ibáñez *et al.*, 2006) have suggested its presence to be restricted to the northern part of Iberia, although it could potentially occupy other parts of Iberia given that in other countries it was also found in more xeric areas (Juste *et al.*, 2013). As *E. isabellinus* is a recently discovered species in Iberia, little is known of its actual distribution. However, (García-Mudarra *et al.*, 2009) suggested that *E. isabellinus* occupied the southern part of Iberia in allopatry with *E. serotinus*. Our results indicate that *E. isabellinus* may have a strong Mediterranean affinity, as it is predicted to occur mainly in southern Iberia where the Mediterranean climate is dominant, which would explain the relevance of climatic variables in this species' distribution models. In fact, a strong connection between Iberian and North African populations of *E. isabellinus* is shown by (Juste *et al.*, 2009). However, the two *Eptesicus* species have extensive niche overlap, indicating that they explore very similar conditions, although geographically only moderate overlap occurs (with the main contact zone in central Iberia). This suggests that each species thrives in a specific biogeographical zone, *E. serotinus* in Atlantic climates and *E. isabellinus* in Mediterranean areas, but considering the large niche overlap it is quite possible that competition may occur if prey resources are limited in contact zones. In fact, it was shown by (Brito *et al.*, 2009) with North African canids of the genus *Vulpes* that species with different biogeographical affinities but with overlap in habitat requirements may be suffering from spatial exclusion through competition. (Bilgin *et al.*, 2008) demonstrated that two morphologically similar species of *Miniopterus* bats were separated by climatic affinities. Later, (Furman *et al.*, 2010) showed that the geographical distributions of these species were the result of historical and ongoing expansion events, and interlineage competition rather than the outcome of climatic preferences. As such, only through future

phylogeographical analyses and studies directed to habitat use and resource selection will it be possible to clarify this situation.

Limitations and caveats

When considering genetic methodology, we are aware of the possible limitations or errors of sequencing only mitochondrial DNA (Zhang & Hewitt, 2003). However, our samples were sequenced using markers developed by (Ibáñez *et al.*, 2006) that have been tested numerous times, which guarantee the correct identification of the sampled individuals.

To develop adequate prediction using distribution models, it is advisable to have the best sampling coverage of each species' distribution (Jiménez-Valverde *et al.*, 2008). Moreover, it is quite frequent that species distribution modelling studies highlight new areas of occurrence (e.g. (Raxworthy *et al.*, 2003; Rebelo & Jones, 2010). In this study, the long-term sampling effort over several years ensured almost complete coverage of the Iberian Peninsula (the exception being the north–central region of the Cantabrian mountain ridge in Spain). By comparing distribution models with the sampling coverage there was a strong indication that each species' realized distribution was covered. However, some isolated patches of suitability were predicted by the models, in extreme areas of species' distributions. These areas are known to be out of the distribution limits of the species; for example, *P. auritus* has not been found in the Balearics nor *M. mystacinus* in the mountains of Andalucia. These areas could have been beyond the dispersion capacity of the species.

Finally, when analysing possible ecological niches of the species and the interactions between them through ecological niche modelling, we must take into account the resolution of the variables and the scale of the analyses (Soberón & Nakamura, 2009). Biotic interactions such as competition or resource availability are generally studied at local scales and are barely considered at larger scales (Whittaker *et al.*, 2001). Thus, we propose only possible interactions that may occur between the studied species within each complex, according to realized niche as delimited by the resolution of our models. These are hypotheses that need further study at local scales within each of the contact zones for each species complex.

Conclusions

The analyses of species distributions and ecological niches allowed us to differentiate several possible interactions occurring within the cryptic complexes studied. We observed niche

overlap between species occupying similar biogeographical regions (the *Plecotus* and *Myotis* complexes), and between species occupying different biogeographical regions (the *Eptesicus* complex). First, in the *Plecotus* complex distributions could be explained by competitive exclusion. The *Myotis* complex showed considerable overlap in niches and distributions between species, suggesting stable coexistence by resource partitioning. Finally, in the *Eptesicus* complex there is a higher uncertainty as to what is occurring, as there was a surprisingly significant overlap between both species' ecological niches, but a relatively small overlap in space, resulting in a few contact zones. This could be a phenomenon of competitive exclusion where the niches have stabilized, but their overlap can also imply that the species' niches are still evolving and that competition is actively occurring.

When analysing the biogeographical patterns of the contact zones, we see a clear association of the contact zones of the *Plecotus* and of the *Myotis* complexes to the Eurosiberian regions. The contact zone of the *Eptesicus* complex seems to be in the areas of transition between the Eurosiberian and Mediterranean biogeographical zones, which are associated with the affinities of *E. serotinus* and *E. isabellinus*, respectively.

This study brings relevant insight into the distributions and biogeographical affinities of cryptic species, allowing the determination of contact zones within complexes. It highlights the importance of how different interactions within complexes can influence each species' ecological niche and, consequently, its geographical distribution. Determining cryptic species' distributions and their ecological requirements and interactions will aid in conservation management and with establishment of the conservation status of the newly discovered species.

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SUPPORTING INFORMATION I

Appendix S1 - Genetic methodology

Genomic DNA was extracted from half of a 3 mm wing punches using an E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, GA, USA), eluted and stored in 50 µl of the provided elution buffer. One mitochondrial fragment was amplified: the cytochrome-b (cyt b). To amplify this fragment, we used the following sets of primers: Molcit-F (Ibáñez *et al.*, 2006) and MVZ-16 (Smith & Patton, 1993). Polymerase chain reaction (PCR) for cyt b was performed with 5µl of MasterMix (Quiagen Multiplex PCR Kit), 0.4 µl of each primer (at 10 pmol), 3.2 µl of H_2O and 1 µl of genomic DNA. The amplification consisted of an initial denaturation at 95°C for 15 min, followed by 40 cycles at 90°C for 30 s, 50°C for 45 s, and 72°C for 1 min, with a final elongation step at 60°C for 10 min. The amplified fragments of cyt b were sequenced on an automated sequencer (ABI 310; Applied Biosystems) in both directions using the same primers. Sequences from the fragment were examined, edited and aligned using the software Bioedit v.7.0.1. Obtained sequences were then compared with those available at GenBank. Species identification was done using the application Nucleotide Blast (NCBI GenBank), in a similar approach to Medinas *et al*. (2013).

Sequences were aligned in Genious 4.8.5. (Biomatters, New Zealand), using the CLUSTAL W option (Larkin *et al*., 2007) and by visual inspection. The considered outgroup was *Pipistrellus pipistrellus* (Genbank accession number: DQ120854). All new haplotype sequences obtained in this study were submitted to GenBank (Table S1.1 - S1.3.).

Table S1.1. GenBank accession numbers of the cytocrome b sequences used in this study for *P. auritus/begognae*.

Species	**Haplotype**	**GenBank number**	**Location**
P. auritus	PA1	AF513768	Switzerland
P. auritus	PA2	AF513765	Iberia
P. auritus	PA3	AY306211	Iberia
P. auritus	PA4	AF513760	Iberia
P. auritus	PA5	AF513769	Austria
P. auritus	PA6	AF513756	Denmark
P. begognae	PB1	AF513761	Iberia
P. begognae	PB2	AF513762	Iberia
P. begognae	PB3	AF513767	Iberia
P. begognae	PB4	AF513764	Iberia

Table S1.2. – GenBank accession numbers of the cytocrome b sequences used in this study for *M. mystacinus/alcathoe*.

Species	Haplotype	GenBank number	Localition
M. alcathoe	MA1	DQ120882	Iberia
M. alcathoe	MA2	DQ120883	Iberia
M. alcathoe	MA3	JX258121	Croatia
M. alcathoe	MA4	EU795690	Czech Republic
M. mystacinus	MM1	DQ120881	Iberia
M. mystacinus	MM2	DQ120880	Iberia
M. mystacinus	MM3	EU360643	Iberia
M. mystacinus	MM4	AF376861	Germany
M. mystacinus	MM5	AY665140	Czech Republic
M. mystacinus	MM6	DQ120879	Iberia

Table S1.3. – GenBank accession numbers of the cytocrome b sequences used in this study for *E. serotinus/isabellinus*.

Species	Haplotype	GenBank number	Location
E. serotinus	ES1	EU786845	Denmark
E. serotinus	ES2	DQ120836	Iberia
E. serotinus	ES3	EU786844	Czech Republic
E. serotinus	ES4	KF218369	Turkey
E. serotinus	ES4	DQ120834 - DQ120835	Iberia
E. serotinus	ES4	EU786863	United Kingdom
E. serotinus	ES4	EU786852	Iberia
E. serotinus	ES4	EU786848	Italy
E. serotinus	ES4	EU786846	France
E. serotinus	ES4	KJ710485*	Iberia
E. serotinus	ES5	DQ120833	Iberia
E. serotinus	ES5	EU786853	Iberia
E. serotinus	ES6	DQ120832	Iberia
E. serotinus	ES6	EU786854	Iberia
E. serotinus	ES7	KJ710486*	Iberia
E. isabellinus	EI1	EU360696	Iberia
E. isabellinus	EI2	KJ710487*	Iberia
E. isabellinus	EI3	EU360699	Iberia
E. isabellinus	EI3	KJ710488*	Iberia
E. isabellinus	EI4	EU786838	Iberia
E. isabellinus	EI5	KJ710489*	Iberia
E. isabellinus	EI6	KJ710490*	Iberia
E. isabellinus	EI7	EU786837	Iberia
E. isabellinus	EI7	EU786836	Iberia
E. isabellinus	EI7	KJ710491*	Iberia
E. isabellinus	EI8	EU360694	Iberia
E. isabellinus	EI9	EU360692	Iberia
E. isabellinus	EI10	EU360693	Iberia
E. isabellinus	EI11	EU360691	Iberia
E. isabellinus	EI12	KJ710492*	Iberia
E. isabellinus	EI13	EU360691	Iberia
E. isabellinus	EI14	EU360695	Iberia
E. isabellinus	EI15	KJ710493*	Iberia
E. isabellinus	EI16	KJ710494*	Iberia

*New haplotypes from this study

Appendix S2 - Phylogenetic analyses

Methodology

We used MEGA 5.2 (Tamura *et al*., 2011) to generate a maximum-likelihood tree of the phylogeny of each cryptic complex. The tree was built using haplotypes generated for each cryptic complex in DnaSP (Rozas *et al*., 2003). The substitution model for each complex was selected using AIC implemented in jModelTest 2.1.4 (Darriba *et al*., 2012). The best tree was obtained using a heuristic search with the nearest-neighbour interchange algorithm. Branch support for the phylogenetic tree was determined through boostrap replicates.

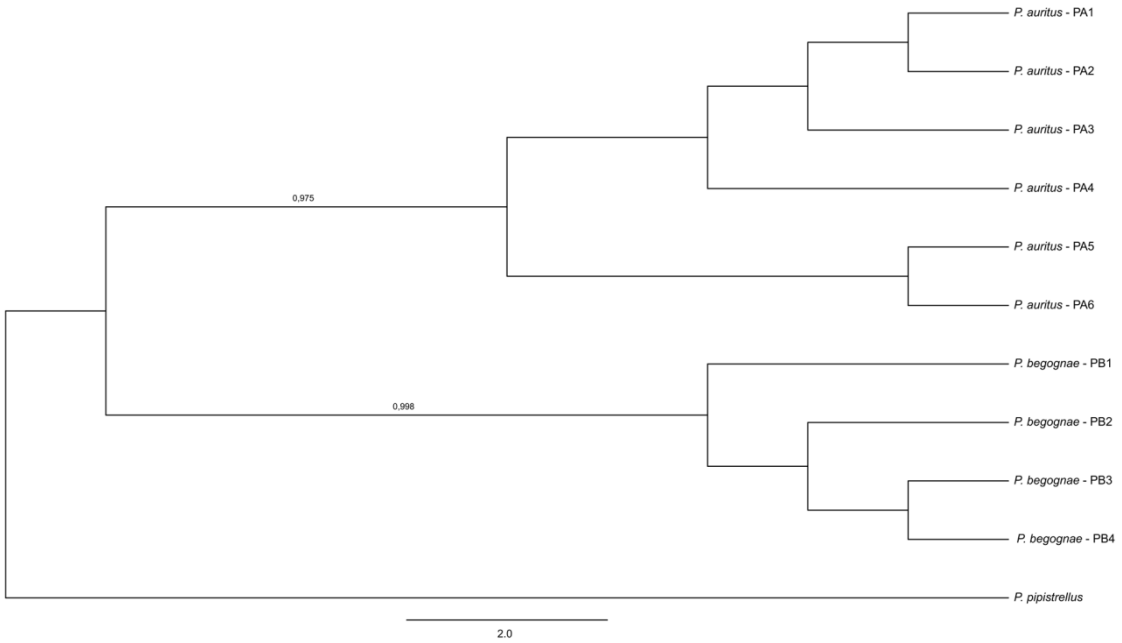

Fig. S2.1. Maximum-likelihood phylogenetic tree for the complex *P. auritus/begognae*.

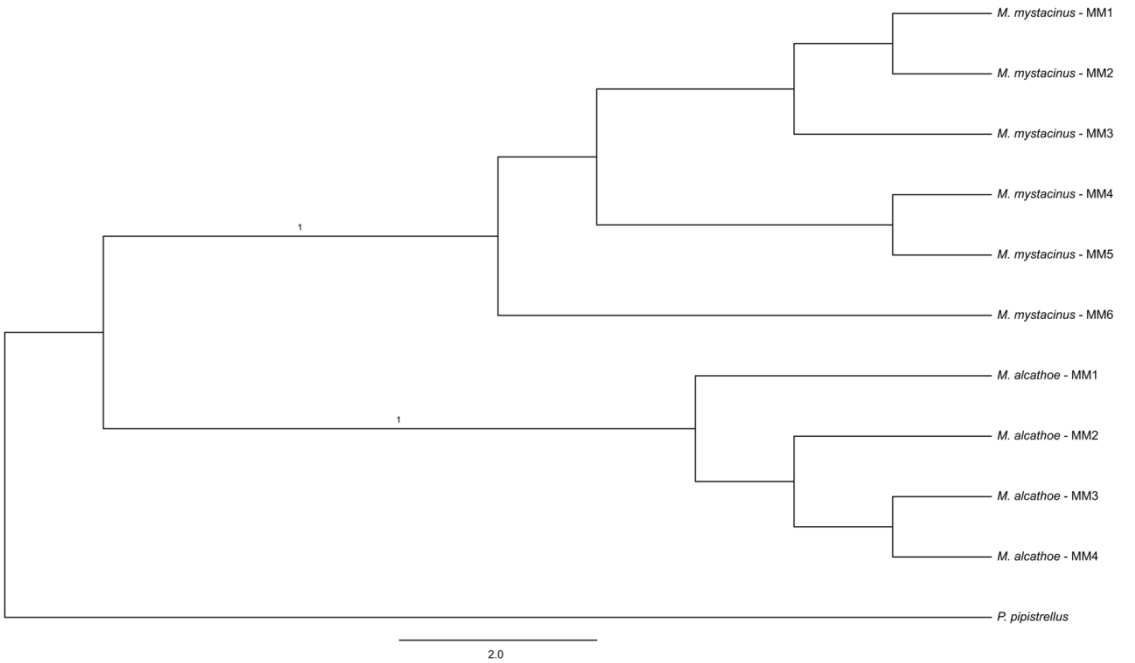

Fig. S2.2. Maximum-likelihood phylogenetic tree for the complex *M. mystacinus/alcathoe*.

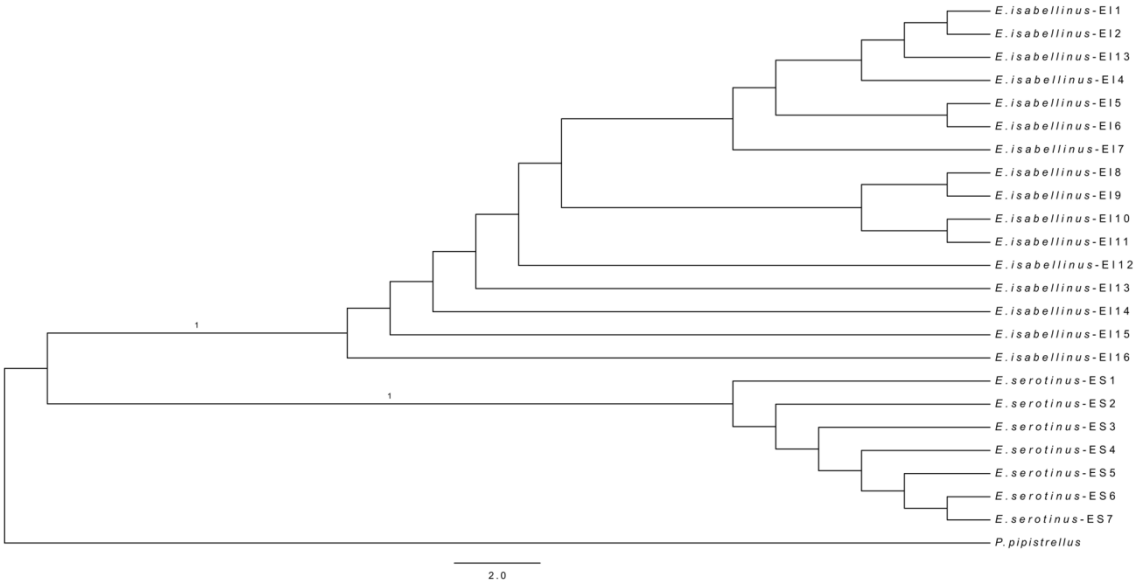

Fig. S2.3. Maximum-likelihood phylogenetic tree for the complex *E. serotinus/isabellinus.*

Appendix S3 - Ecogeographical variables and variable importance

Table S3 - The initial set of 45 ecogeographical variables.

Type	Variable		Code	Source
Climatic	Annual Mean Temperature		ib_bio1	WorldClim (~1950-2000)
	Mean Diurnal Range		ib_bio2	
	Isothermality		ib_bio3	
	Temperature Seasonality		ib_bio4	
	Max Temperature of Warmest Month		ib_bio5	
	Min Temperature of Coldest Month		ib_bio6	
	Temperature Annual Range		ib_bio7	
	Mean Temperature of Wettest Quarter		ib_bio8	
	Mean Temperature of Driest Quarter		ib_bio9	
	Mean Temperature of Warmest Quarter		ib_bio10	
	Mean Temperature of Coldest Quarter		ib_bio11	
	Annual Precipitation		ib_bio12	
	Precipitation of Wettest Month		ib_bio13	
	Precipitation of Driest Month		ib_bio14	
	Precipitation Seasonality		ib_bio15	
	Precipitation of Wettest Quarter		ib_bio16	
	Precipitation of Driest Quarter		ib_bio17	
	Precipitation of Warmest Quarter		ib_bio18	
	Precipitation of Coldest Quarter		ib_bio19	
Topographical	Altitude		ib_dem	SRTM (CGIAR-CSI 2008)
	Mean slope		ib_slope	
	Maximum slope		ib_slopemax	
	Mean slope >10°		ib_sl10	
	Mean slope >15°		ib_sl15	
	Mean slope >20°		ib_sl20	
	Maximum slope >1°		ib_slmax1	
	Maximum slope >3.5°		ib_slmax35	
	Maximum slope >7°		ib_slmax7	
	Maximum slope >10°		ib_slmax10	
	Maximum slope >15°		ib_slmax15	
	Maximum slope >20°		ib_slmax20	
	Distance to slope >10°		ib_di_sl10	
	Distance to slope >15°		ib_di_sl15	
	Distance to slope >20°		ib_di_sl20	
	Distance to maximum slope >1°		ib_di_slmax1	
	Distance to maximum slope >3.5°		ib_di_slmax35	
	Distance to maximum slope >7°		ib_di_slmax7	
	Distance to maximum slope >10°		ib_di_slmax10	
	Distance to maximum slope >15°		ib_di_slmax15	
	Distance to maximum slope >20°		ib_di_slmax20	
Habitat	Land cover:	Agriculture 1 Orchards 2 Forested agriculture 3 Forest 4 Coniferous 5 Shrubs 6 Bare 7 Urban 8 Water 9 Eucalyptus plantations 10	ib_land	GLOBCOVER (2006)
	Distance to forests		ib_di_forest	
	Distance to agriculture		ib_di_agric	
	Distance to water bodies		ib_di_water	
	Distance to eucalyptus plantations		ib_di_eucal	

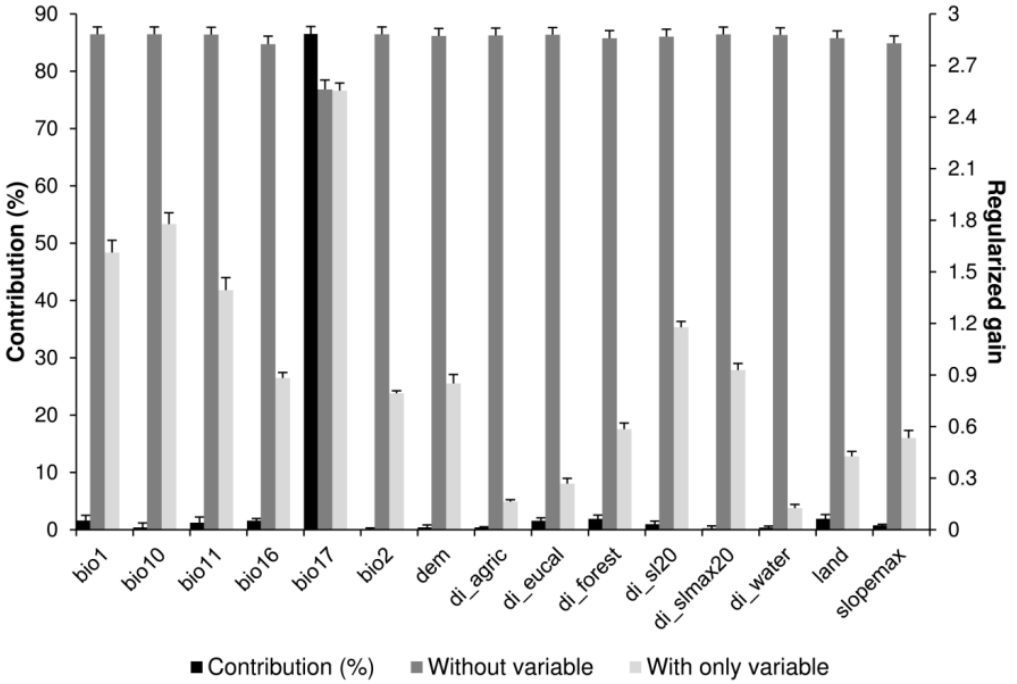

Figure S3.1. - Graph representing variable importance for *Plecotus auritus* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

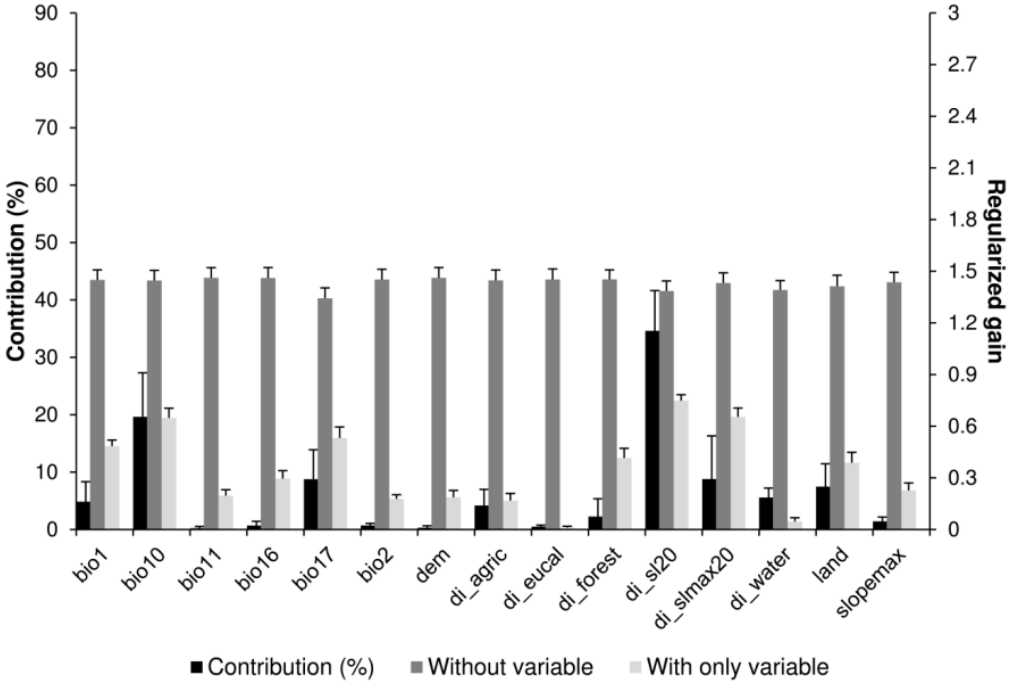

Figure S3.2. - Graph representing variable importance for *Plecotus begognae* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

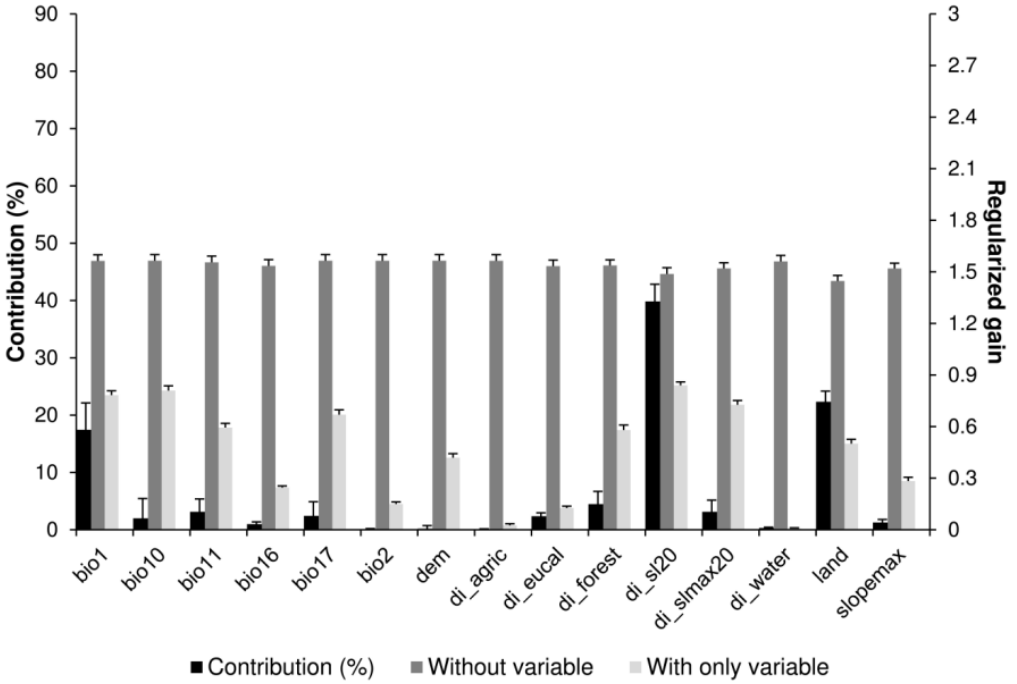

Figure S3.3. - Graph representing variable importance for *Myotis mystacinus* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

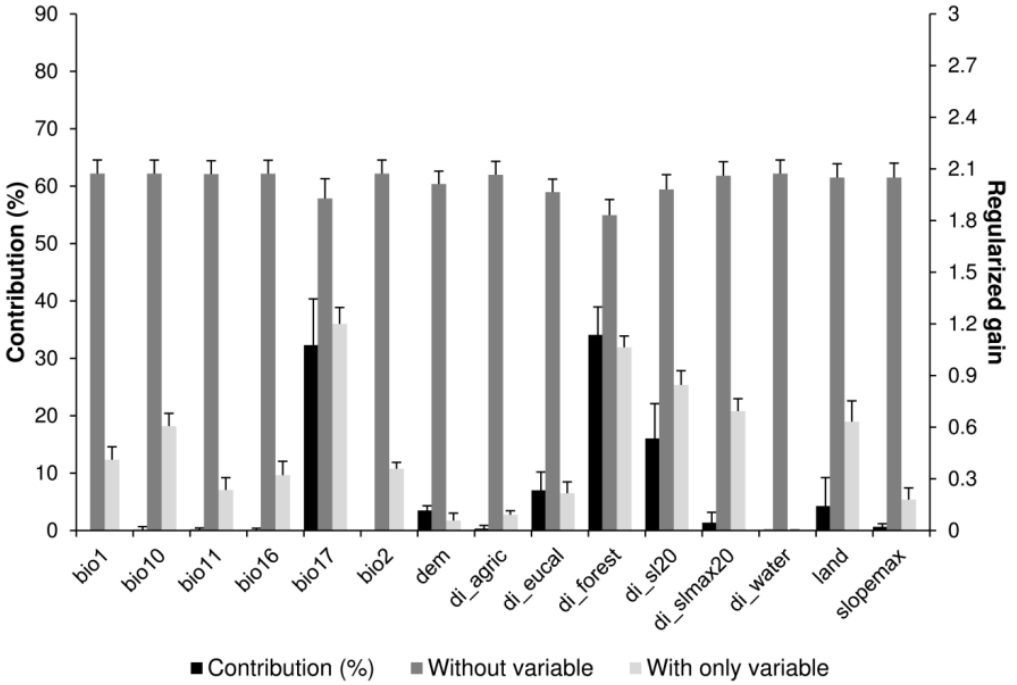

Figure S3.4. - Graph representing variable importance for *Myotis alcathoe* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

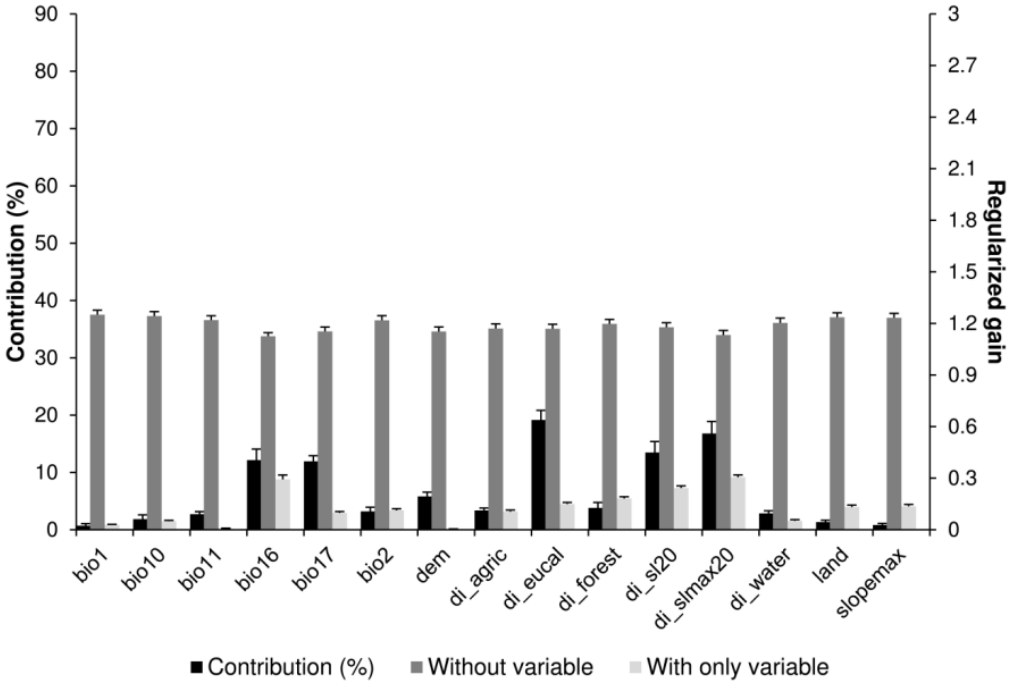

Figure S3.5. - Graph representing variable importance for *Eptesicus serotinus* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

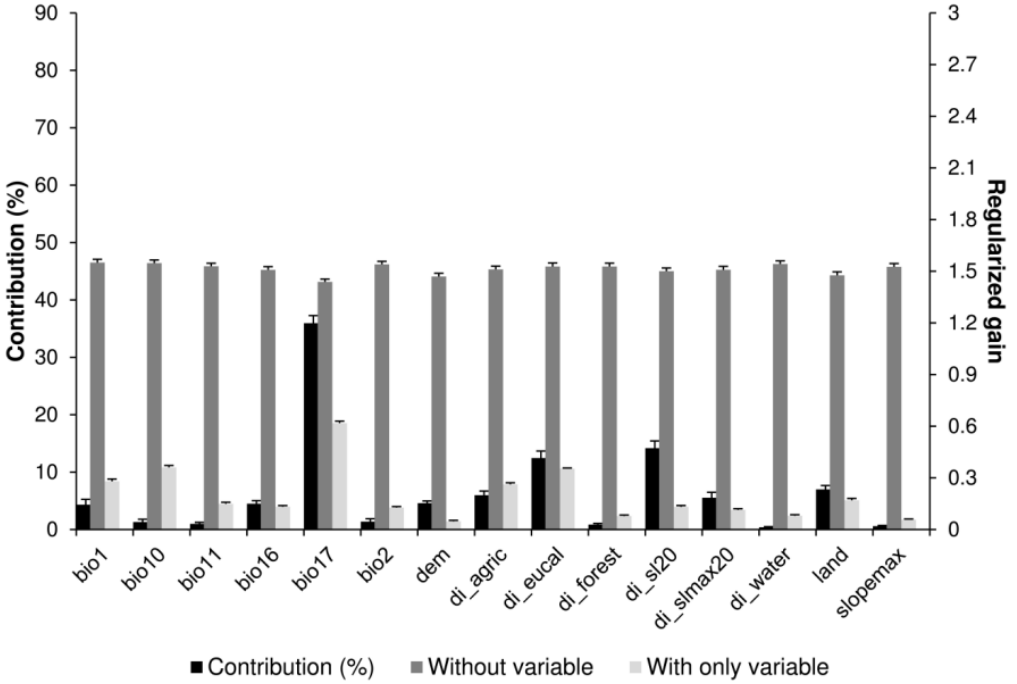

Figure S3.6. - Graph representing variable importance for *Eptesicus isabellinus* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S3.

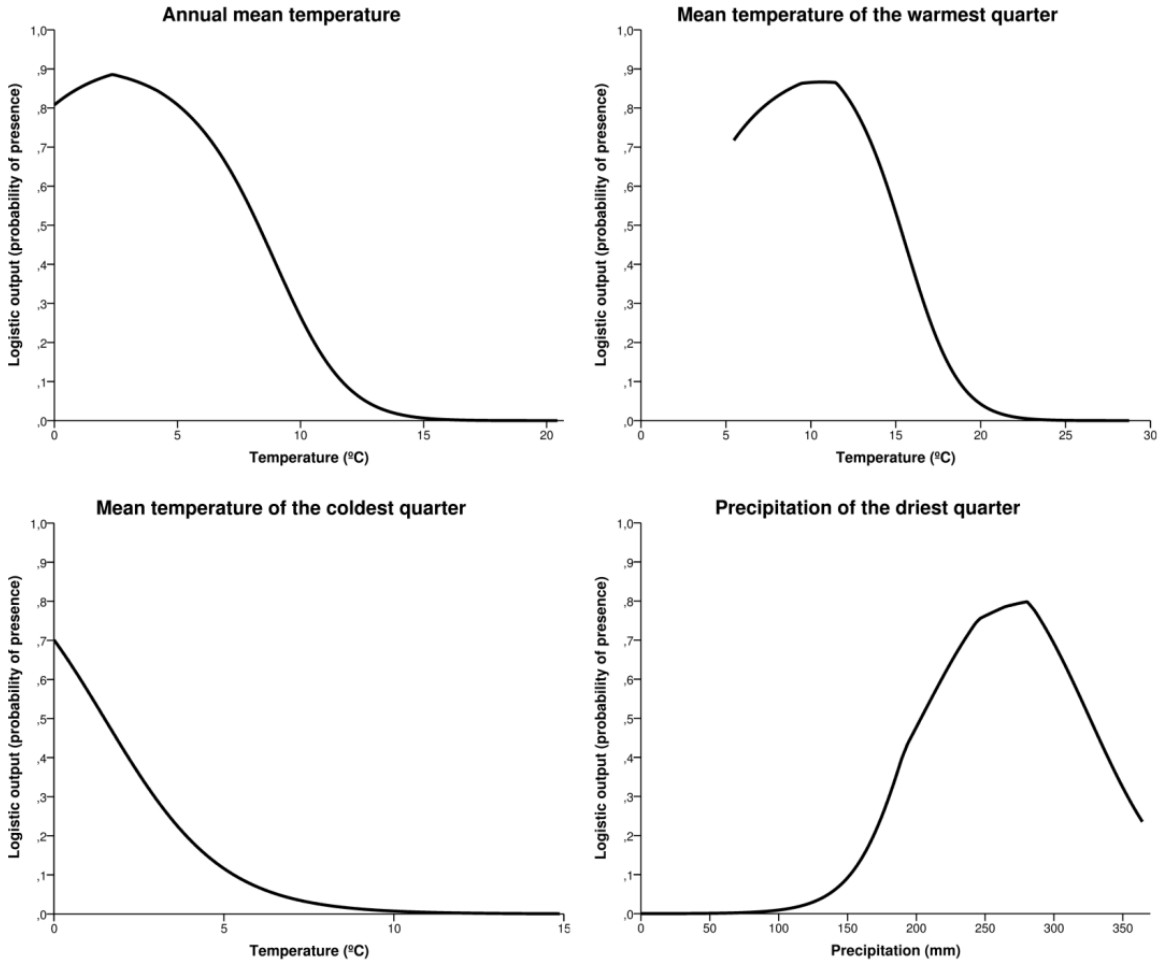

Figure S3.7. - Response curves of the environmental variables most related to the distribution of *Plecotus auritus*.

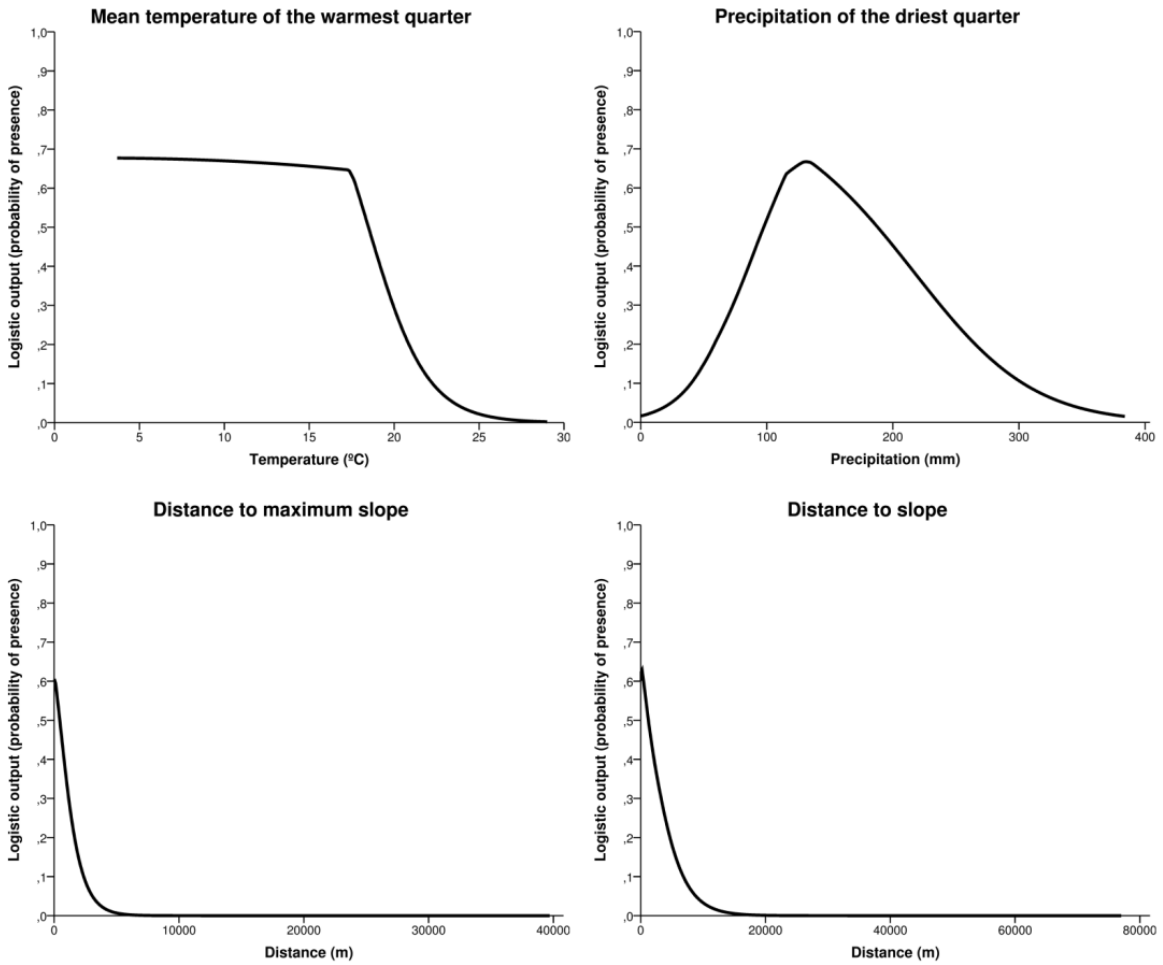

Figure S3.8. - Response curves of the environmental variables most related to the distribution of *Plecotus begognae*.

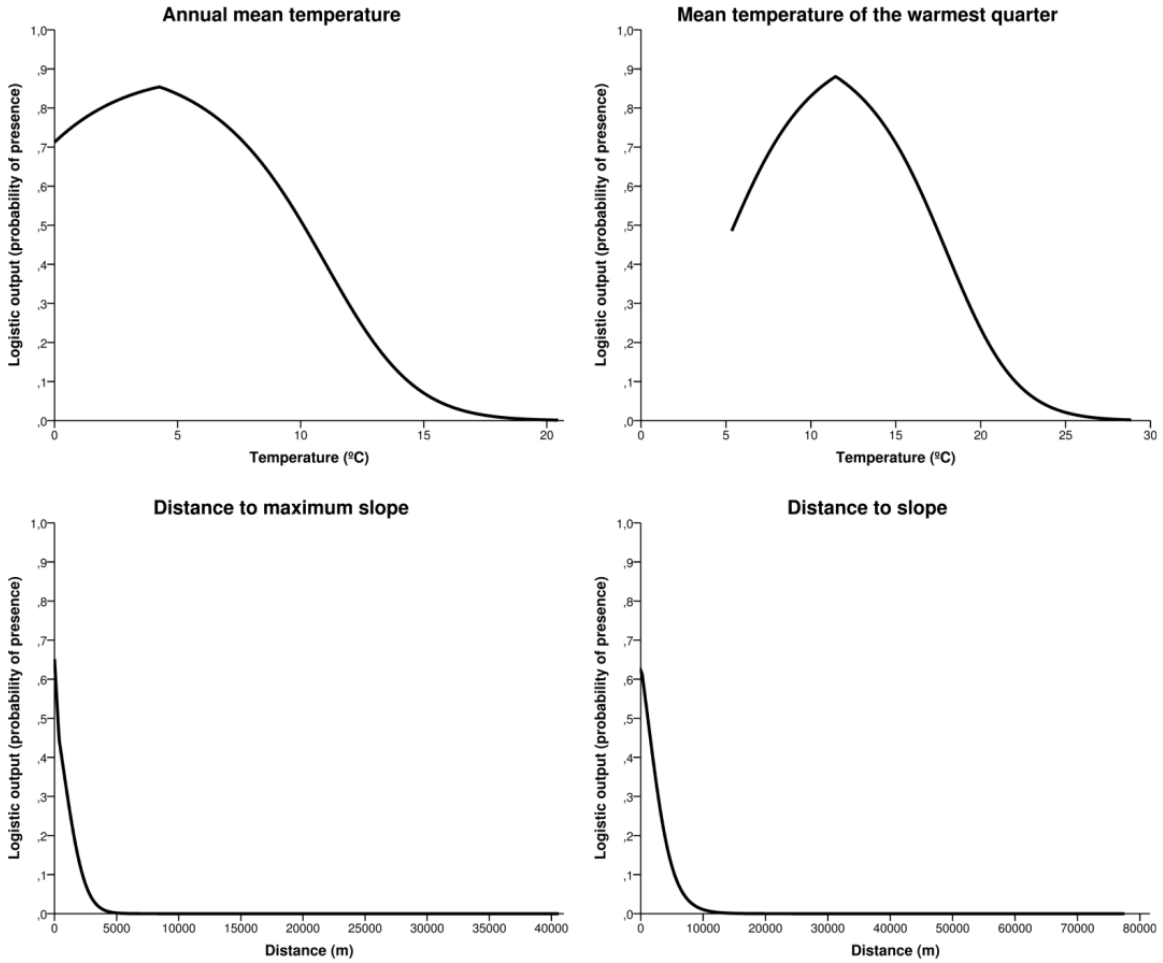

Figure S3.9. - Response curves of the environmental variables most related to the distribution of *Myotis mystacinus*.

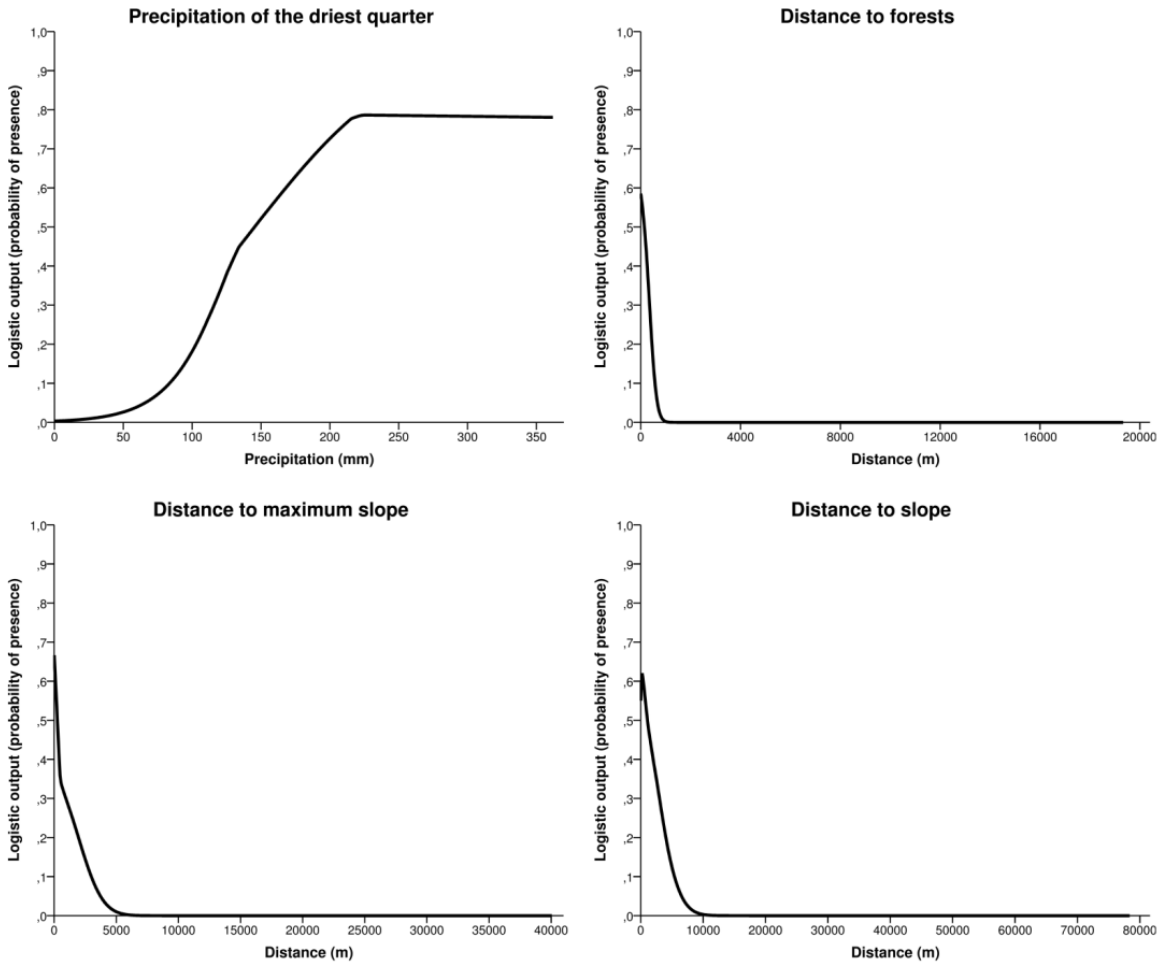

Figure S3.10. - Response curves of the environmental variables most related to the distribution of *Myotis alcathoe*.

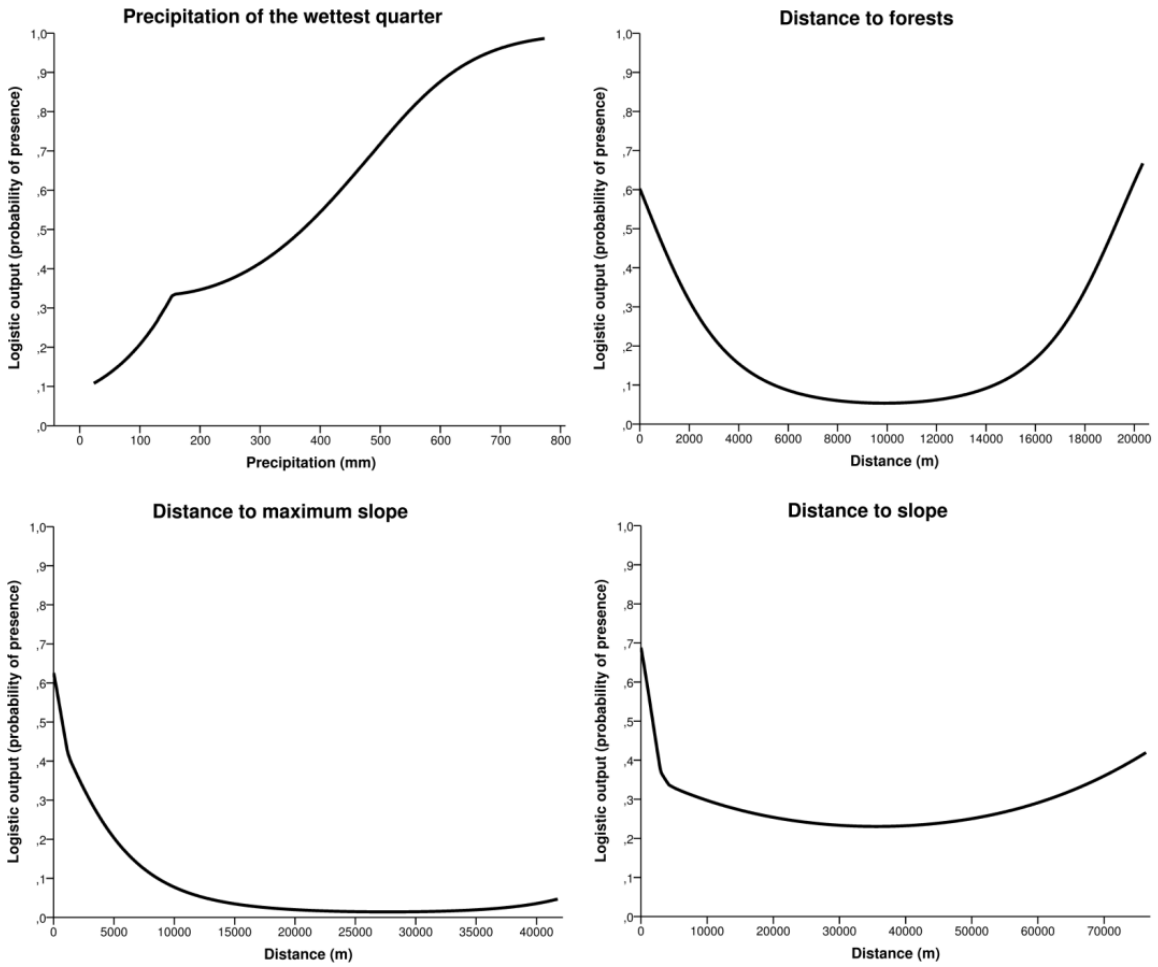

Figure S3.11. - Response curves of the environmental variables most related to the distribution of *Eptesicus serotinus*.

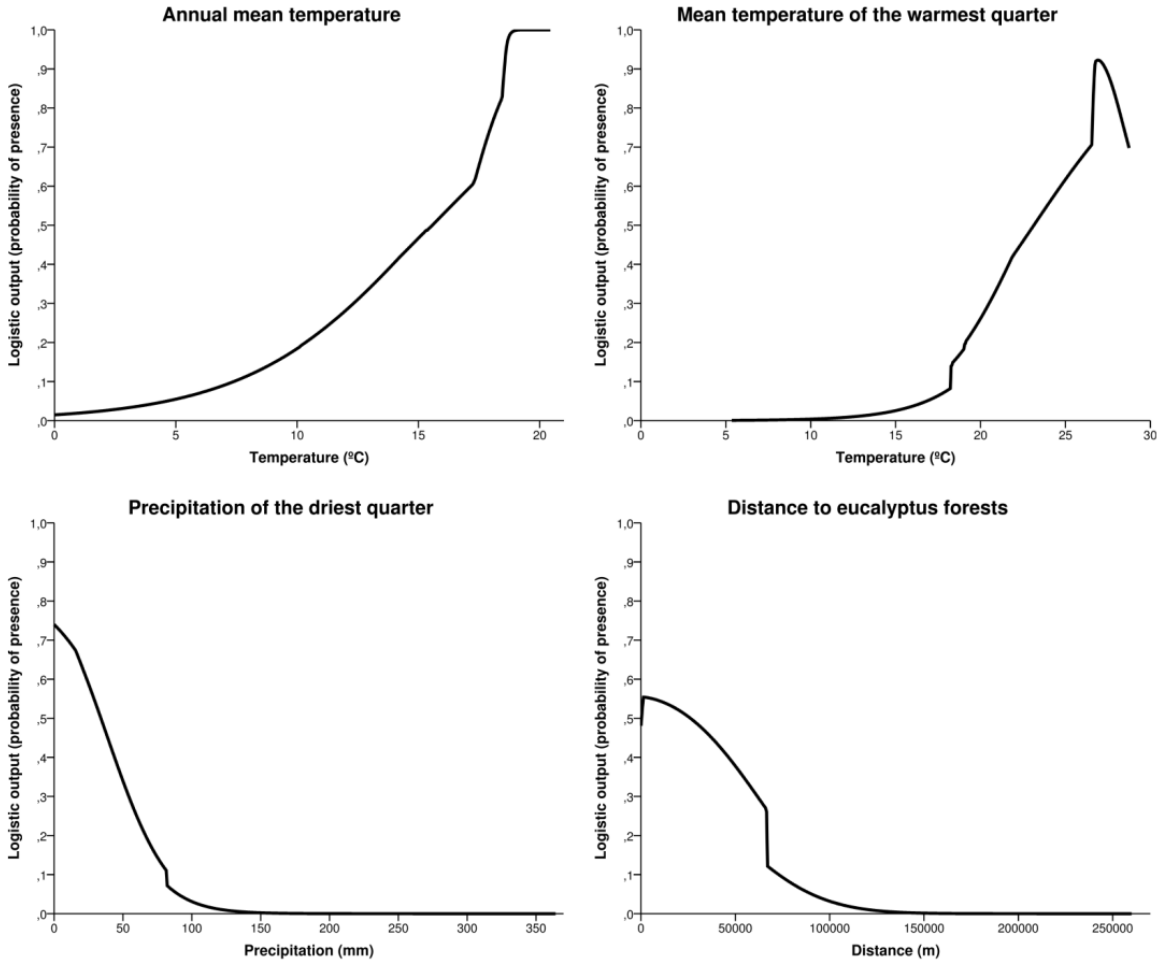

Figure S3.12. Response curves of the environmental variables most related to the distribution of *Eptesicus isabellinus*.

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CHAPTER 3

EVOLUTIONARY HISTORY OF THE LONG-EARED BATS IN THE IBERIAN PENINSULA

PAPER II - A KNOT IN THE EARS: DISENTANGLING THE EVOLUTIONARY HISTORY OF LONG-EARED BATS (*PLECOTUS* SPP.) IN THE IBERIAN PENINSULA

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ABSTRACT

Due to increasingly rapid DNA sequencing and the advances in molecular methods over the past decades, several cryptic species have recently been identified. In fact, 20% of Iberian bat species have shown to harbour complexes of cryptic species, most likely due to this area's rich genetic diversity, as it was a major glacial refugium for several taxa. Along with numerous others, two new cryptic bat lineages of the genus *Plecotus* have been identified in the Iberian Peninsula, *Plecotus macrobullaris* and *Plecotus auritus begognae*. This work aims at elucidating the evolutionary history and phylogenetic relationships of the bats of the genus *Plecotus* in Iberia, particularly focusing on the poorly-known lineage *P. a. begognae*. A multi-marker approach was used, integrating mtDNA, nDNA and microsatellites, to develop phylogenetic reconstructions and combine them with the genetic structure of the lineages, therefore inferring on the phylogenetic history and, ultimately, the taxonomic status of *Plecotus* spp. in Iberia. All markers used showed a clear distinction of *Plecotus austriacus* and *Plecotus macrobullaris* in separate phylogenetic clades and microsatellite clusters. However, the separation between the lineages *Plecotus auritus auritus* and *P. a. begognae* was only identified in the mtDNA phylogenetic reconstruction and microsatellite clusters, demonstrating slight gene flow patterns. The results obtained therefore suggest a possible past isolation of *P. a. begognae* within Iberia (probably occurring during the last glacial period), separated from *P. a. auritus*, and after recently coming into contact, gene flow between the two lineages seems to be occurring, although at very low levels. As such, our results seem to support the identification of *P. a. begognae* as an Iberian subspecies of *P. a. auritus*.

INTRODUCTION

The advances in molecular phylogenetic methods and their application in evolutionary studies over the past decades, have given significant insights on taxonomy and promoted the discovery of a great number of species for science (Bickford *et al.*, 2006). This phenomenon has also hallowed the discovery of several new cryptic species worldwide (Hebert *et al.*, 2004; Chadès *et al.*, 2008; Kaliontzopoulou *et al.*, 2011; Boratynski *et al.*, 2012; Nair *et al.*, 2012). These newly found cryptic complexes, ecologically and/or genetically distinct species with very similar morphology (Jones, 1997), bring the need for new biodiversity assessments, since they may represent rare or threatened taxa, requiring re-evaluations on their distribution, ecological requirements and conservation status (Bickford *et al.*, 2006; Sattler *et al.*, 2007). Therefore, understanding cryptic species' evolutionary history and phylogenetic structure becomes an

important tool for the conservation of species' genetic diversity, while also aiding in the identification and management of evolutionary significant units within species (Moritz, 1994).

Bats are among one of the faunal groups where molecular approaches have boosted the number of new species (Mayer & von Helversen, 2001; Ibáñez *et al.*, 2006; Mayer *et al.*, 2007; Sun *et al.*, 2009; Puechmaille *et al.*, 2014), yet controversy on the taxonomic position still persists in a number of cryptic species, due to the wide degree of genetic differentiation found within the cryptic complexes. Particularly in Europe, several cryptic complexes of bat species have been discovered, such as *Myotis mystacinus/alcathoe*, *Eptesicus serotinus/isabellinus* and even possible new species in complexes such as *Myotis nattereri/Myotis escalerai/Myotis spA*, and at a less extent, *Pipistrellus kuhlii* and *Hypsugo savii* (Barratt *et al.*, 1997; Mayer & von Helversen, 2001; Agirre-Mendi *et al.*, 2004; Juste *et al.*, 2004; Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Salicini *et al.*, 2013). Of exceptional interest are the bats of the genus *Plecotus*, while historically, in classic systematics, there were only two main species recognised (*Plecotus auritus* Linnaeus, 1758 and *Plecotus austriacus* Fischer, 1829), recently an astonishing number of new *Plecotus* species and subspecies have been found, all showing extremely similar morphological characteristics (Spitzenberger *et al.*, 2001; Mucedda *et al.*, 2002): Spitzenberger *et al.* (2006) confirmed *Plecotus teneriffae* Barret-Hamilton, 1907, endemic to the Canary Islands; Spitzenberger *et al.* (2003) described *Plecotus macrobullaris* Kuzjakin, 1965 (a synonym of *Plecotus alpinus* Kiefer and Veith, 2002 and *Plecotus microdontus* Spitzenberger 2002), inhabiting mountainous regions from the Caucasus and Asia Minor, to the Balkans, the Alps and the Pyrenees (Kiefer & Veith, 2001; Spitzenberger *et al.*, 2002; Spitzenberger *et al.*, 2003); Mayer & von Helversen (2001) and Kiefer *et al.* (2002) described *Plecotus kolombatovici* Dulic, 1980, from the Balkans; and Mucedda *et al.* (2002) found *Plecotus sardus* Mucedda, Kiefer, Pidinchedda & Veith, 2002, endemic to the island of Sardinia. As outlined, several of the Southern European peninsulas have shown evidence of cryptic speciation in the bats of the genus *Plecotus*. In the Iberian Peninsula, there are three recognised species of this genus, *P. auritus*, *P. austriacus* and *P. macrobullaris*. However, Juste *et al.* (2004) and Ibáñez *et al.* (2006) both provided molecular evidence of possible Iberian cryptic speciation of *P. auritus*, hereafter *P. auritus auritus*, into a previously described lineage *Plecotus a. begognae* (de Paz, 1994). However, the lack of sampling and markers did not allow a full confirmation of the taxonomic status of the lineage described as subspecies of *P. a. auritus* in the study of de Paz (1994). This study (de Paz, 1994) was solely based on morphometric analyses and described *P. a. begognae* as having slightly larger dimensions than *P. a. auritus*, but generally having quite similar morphology, and occurring in the Iberian and Central mountain systems and in the south of the Cantabrian Mountains and south of the

Pyrenees. Later, it was found by Santos *et al.* (2014) that this Iberian lineage not only seems to occur in mountainous regions throughout the whole North of the Iberian Peninsula, but also that it may have similar biogeographical affinities and environmental requirements than those of *P. a. auritus*.

The Iberian Peninsula is known to have been a major refugium for several European species, during the Pleistocene glacial period, as well as a source of northern range colonization during interglacial warmer climatic periods (Hewitt, 2000). This resulted in an important and unique signature of rich evolutionary diversity, reflected in deep genetic divergence within refugial populations (Hewitt, 2004; Hampe & Petit, 2005), therefore higher species richness (Araújo *et al.*, 2008). In fact, several studies have showed the importance of Iberia as a glacial refugium and source of evolutionary divergence for bats, by demonstrating that it also presented a 'refugia within refugium' pattern, highlighting the evolutionary importance of this peninsula (Rebelo *et al.*, 2012; Dool *et al.*, 2013; Razgour, 2015). This concept supports the occurrence of several refugia within the Iberian Peninsula, reflected in still persisting complex patterns of population genetic structure of lineages which may have remained isolated inside the peninsula by the Pyrenees mountain range (Gómez & Lunt, 2007).

In this study, several records of the different *Plecotus* lineages from the Iberian Peninsula were used to infer on the evolutionary relationships within these main Iberian lineages of long-eared bats, with a particular focus on the cryptic complex *P. a. auritus/P. a. begognae*. In order to obtain a higher evolutionary and phylogenetic resolution, we analysed an array of different mtDNA and nDNA markers, including microsatellites. By combining markers with different evolutionary rates, phylogenetic relationships are able to be analysed at different evolutionary time scales, giving better insights on both phylogenetic and evolutionary structures (Juste *et al.*, 2013; Razgour, 2015). Therefore, we aimed at clarifying the evolutionary history and phylogenetic relationships of the Iberian long-eared bats, providing further evidence on the evolutionary differentiation and, ultimately, taxonomic status of the lineage *P. a. begognae*.

MATERIALS AND METHODS

Study area and samples

The study area was centred in the Iberian Peninsula, located in south-western Europe. It is characterised by a complex topography and unique positioning between the Mediterranean Sea and the North Atlantic, creating two distinct bioclimatic regions, Eurosiberian and Mediterranean (Gómez & Lunt, 2007; Sillero *et al.*, 2009; Romo & García-Barros, 2010). A total of 47 samples of the Iberian *Plecotus* lineages were first considered in the phylogenetic study, afterwards 153 samples were added for the microsatellite analyses (Fig. S1.1, Appendix S1, Supplementary information II). These were obtained from mist netting and roost trapping sessions throughout the Iberian Peninsula (Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Rebelo *et al.*, 2012). A tissue sample was collected from each specimen, through a 3 mm biopsy punch in the wing membrane, and stored in ethanol for laboratory analyses. For hierarchical outgroup comparison, we used *Barbastella barbastellus* (Spitzenberger *et al.*, 2001; Kiefer *et al.*, 2002).

Genetic methodology and genetic markers

We extracted total genomic DNA from tissue samples using the QIAamp DNA Micro Kit (QIAGEN). A mitochondrial (mtDNA) cytochrome c oxidase subunit I (COI) gene was amplified and sequenced using primers VF1 and VR1 (Ivanova et al. 2006) and four nuclear introns fragments (ABDH11, ACPT, ACOX2 and ROGDI) were amplified and sequenced using primers given by Salicini *et al.* (2011). Amplifications were performed using reaction and thermocycling conditions described in Appendix S2, Supplementary information II. Amplicons were sequenced for both strands following the BigDye Terminator v3.1 Cycle sequencing protocol (Applied Biossystems) and sequencing products were separated in an ABI 3130xl Genetic Analyzer. Sequences were aligned using Geneious 9.0.4 (Kearse *et al.*, 2012) and corrected manually. Alignments were comprised of 568 bp (COI), 382 bp (ABDH), 491 bp (ACOX2), 254 bp (ACPT) and 483 bp (ROGD). For nuclear fragments, we considered nucleotide ambiguities with similar peak size in chromatograms as heterozygous positions. Sequences were phased using the algorithm of Stephens *et al.* (2001) available in DnaSP v.5 (Librado & Rozas, 2009).

Phylogenetic analyses

In order to understand the evolutionary history and relationships between the Iberian *Plecotus* lineages, phylogenetic reconstruction analyses were conducted using Neighbor-joining (NJ) (Saitou & Nei, 1987) and Maximum Likelihood (ML) criteria, for all mtDNA and nDNA sequences. In a first approach, the best fitting substitution models were selected for each analysis using the Akaike Information Criterion (AIC) in jModeltest2 (Darriba *et al.*, 2012). All phylogenetic reconstructions and respective genetic distances (levels of genetic differentiation) between groups were performed using MEGA 5.05 (Tamura *et al.*, 2011). Robustness of NJ and MP tree topologies was assessed through bootstrapping (Felsenstein, 1985), with 1000 replicates each. Only bootstrap values P70% indicate sufficiently resolved topologies (Huelsenbeck & Hillis, 1993). Genetic distance values between lineage groups were obtained through sequence's alignments in Geneious 9.0.4 (Kearse *et al.*, 2012), using the percentage of different bases.

Finally, parsimony haplotype networks were constructed for the COI dataset using the median-joining method (Bandelt *et al.*, 1999) as implemented in PopART v1.7 (Leigh & Bryant, 2015), with epsilon set to 0.

Microsatellite analyses

To have a further understanding of the genetic differentiation among and between lineages on a different evolutionary timescale, all samples used in the phylogenetic study, plus more samples subsequently obtained, were genotyped for 23 autosomal microsatellite loci previously developed for *P. austriacus* (n=20; Razgour *et al.*, 2013) and *P. auritus* (n=3; Burland *et al.*, 1998). Three multiplex reactions were performed (markers per multiplex panel, allele range, PCR reaction conditions and PCR cycle programs are outlined in Appendix S2, Supplementary information II). Forward primers were M13-tailed to follow a fluorescent labelling protocol (Blacket *et al.*, 2012) and a negative control was used to monitor possible contaminants. PCR products were separated by size in an ABI3130xl genetic analyser, while alleles were scored against the GeneScan500 LIZ size standard using GENEMAPPER 4.0 (Applied Biosystems). Results' accuracy were measured through re-amplification of 15% random selected samples for each locus (Bonin *et al.*, 2004), resulting in complete concordance among replicates. Four loci were removed from the analyses (Paus10, Paus11, Paus13 and Paus16), since they exhibited low amplification rates. Population structure was then inferred, using the Bayesian clustering analysis implemented in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Falush *et al.*, 2007) for all genotyped samples, allowing K=1 to K=10.

Individual membership proportions (qi) were assessed using the admixture model in 10 independent runs, with 10^6 MCMC iterations, following a burn-in period of 10^5 iterations. Following this procedure, we used STRUCTURE HARVESTER (Earl & VonHoldt, 2012) to determine the number of distinct clusters, based on the number at which the mean likelihood L(K) and Delta K peaked.

RESULTS

Phylogenetic trees

From the total of 47 *Plecotus* samples, only 35 were successfully sequenced for the mtDNA COI fragment, which, unfortunately, did not include any of the *P. macrobullaris* samples. As such, to avoid losing information on the relationships within the *Plecotus* lineages, ten GenBank sequences of *P. macrobullaris* from the Iberian Peninsula (Alberdi *et al*., 2015) were added to the mtDNA alignments. As for the nDNA introns, 37 samples were sequenced for ABDH, 44 for ACOX2, 39 for ACPT and 45 for ROGD. The evolutionary model selected for each fragment was constantly the Jukes-Cantor (JC) model. In the phylogenetic reconstructions, all long-eared bats formed a monophyletic group with the chosen outgroup, however, within this '*Plecotus* group', different topologies were obtained for mtDNA and nDNA fragments. NJ and ML analyses were concordant, however the NJ reconstruction demonstrated a clearer topology, and, as such, only the NJ trees are shown (Fig. 2.1a and 2.1b). Moreover, as equivalent topologies were also found within all nDNA fragments' reconstructions, a concatenated tree was computed (Fig. 2.1b). In both mtDNA and nDNA reconstructions, two major clades emerge from all analyses, with 100% bootstrap values, the one corresponding to the brown long-eared (*auritus*) group and one corresponding to the grey long-eared (*austriacus*) group. All differences within clades below 70% were collapsed, as well as further substructure within lineages. In the mtDNA reconstruction, within the *auritus* group, a clear separation of the *P. macrobullaris* clade is recognised, with genetic differentiation values ranging from 20.34% to 13.38%. With lower levels of differentiation, from 6.51% to 5.46%, a separation of the *P. a. begognae* group from the *P. a. auritus* group can be observed. The samples recognised as *P. a. begognae* seem to form a sister sub-clade to those recognised as *P. a. auritus*. In the nDNA concatenated phylogenetic reconstruction, there is an evident separation of the *P. macrobullaris* clade, however nDNA fragments did not provide sufficient support to separate *P. a. auritus* from *P. a. begognae*, these forming a single clade.

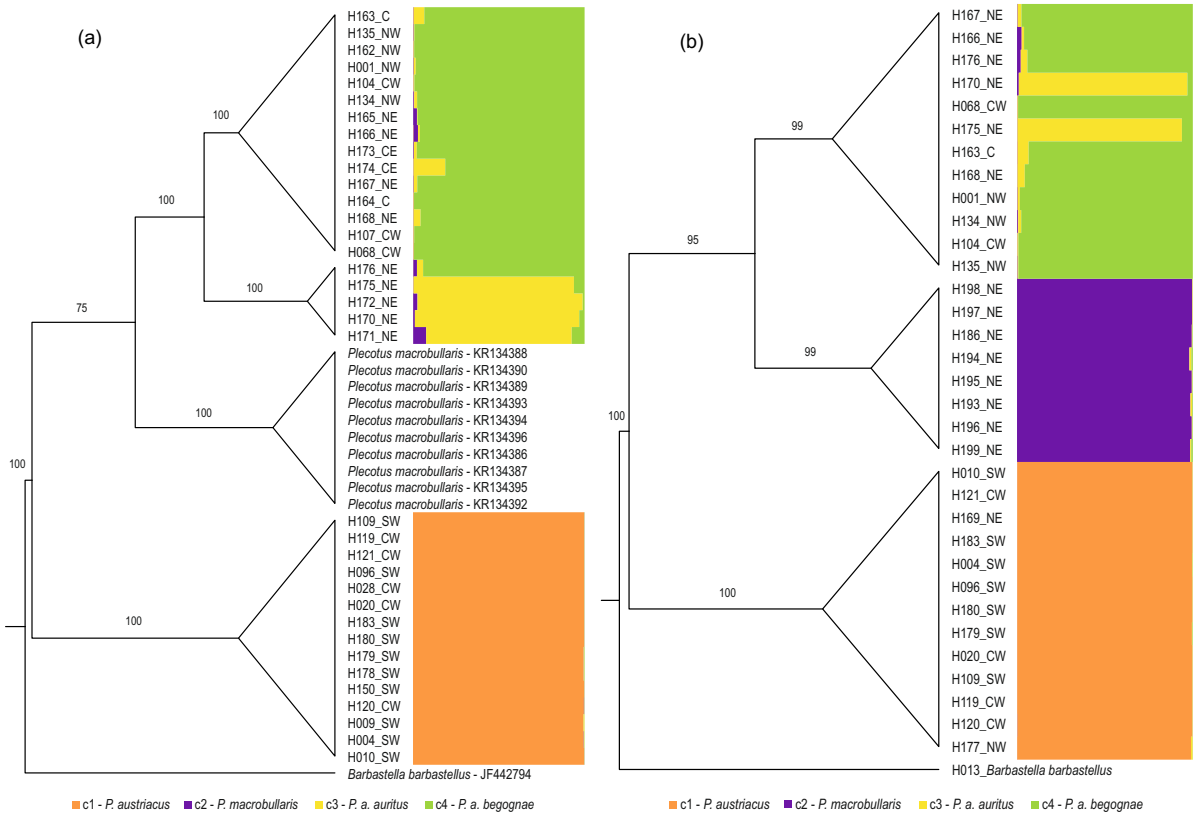

Figure 2.1 - Neighbour-joining tree based on the mitochondrial gene COI (a) and on four nuclear genes, ABDH, ACOX2, ACPT, and ROGD (b), showing the relationship between *Plecotus austriacus*, *Plecotus macrobullaris*, *Plecotus auritus auritus*, and *Plecotus auritus begognae*. Branch values represent bootstrap support. Coloured bars represent the probability of assignment of each individual to a cluster. Location in Iberia is represented: north-west (NW), north-east (NE), central (C), central-west (CW), central-east (CE) and south-west (SW).

Haplotype network analyses

In the haplotype network obtained (Fig. 2.2), a total of 5 different haplotypes were found for the COI fragments of *P. austriacus*, 1 for *P. macrobullaris*, 2 for *P. a. auritus* and 8 *for P. a. begognae*. The network divided the haplotypes into four separate haplo-groups corresponding to each of the *Plecotus* lineages. *P. a. begognae*'s haplotypes were separated from those of *P. a. auritus*' by >18 mutational steps, and from *P. macrobullaris*' and *P. austriacus*' by > 74 and 88 mutational steps, respectively. *P. macrobullaris*' haplotypes were separated from *P. a. auritus*' by >56 mutational steps and from *P. austriacus*' by >162. *P. austriacus*' haplotypes were separated from *P. auritus*' by >106 mutational steps.

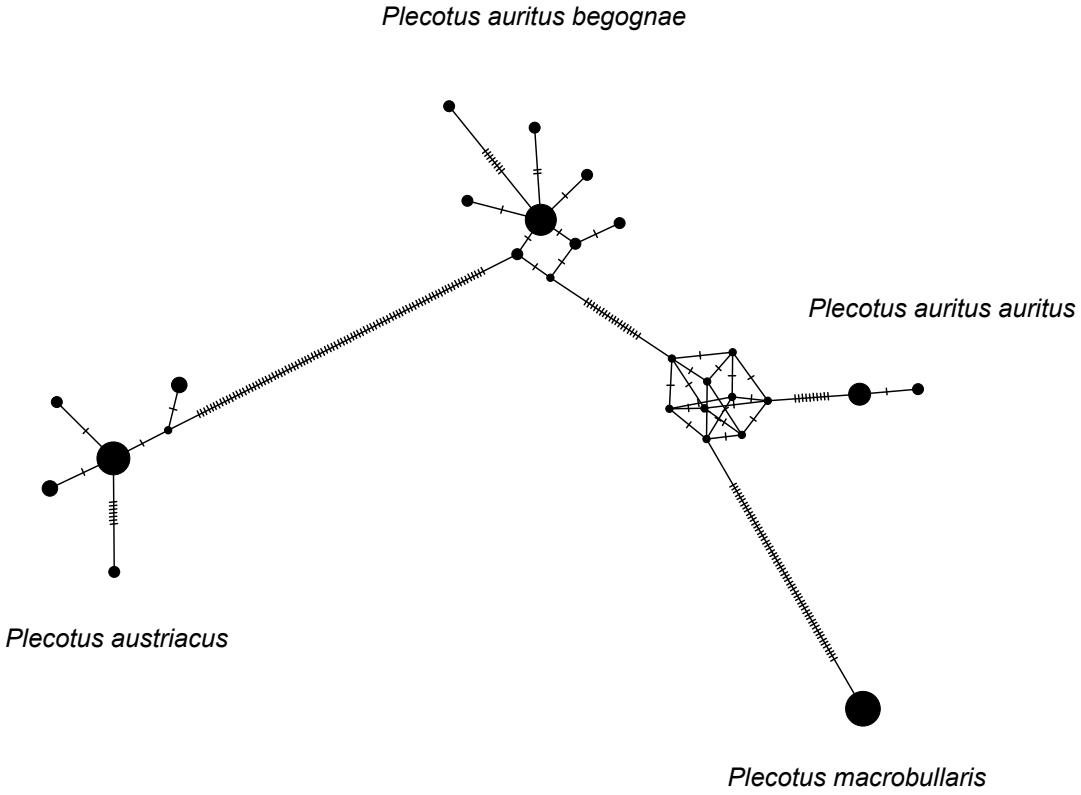

Figure 2.2 - Median joining haplotype network of Iberian *Plecotus* lineages based on mtDNA (COI) sequences. Circles represent haplotypes and are proportional to the number of individuals. Smaller nodes represent intermediate haplotypes not found in this study. Each slash represents a nucleotide substitution.

Microsatellite analyses

The final dataset comprised of 18 loci, as one loci (Paus07) deviated from Hardy–Weinberg equilibrium and was removed from the analyses. Mean likelihood L(K) and Delta K values estimated by STRUCTURE for our dataset, identified four clusters (K=4) as the most likely number of genetic groups in the dataset (Fig. S3.1 and S3.2, Appendix S3, Supplementary information II). Individual assignment values for K=4 (Fig. 2.3) showed a clear separation for *P. austriacus* and *P. macrobullaris* groups. *P. a. auritus* seems to be well separated from the formerly mentioned lineages, showing also a level of differentiation from the *P. a. begognae* group, however with evidence of gene flow. For a more intuitive analysis, individual assignments from the Bayesian clustering analysis are presented together with phylogenetic reconstructions for mtDNA and the concatenated reconstructions from the four nDNA fragments (Fig. 1a and 1b). Microsatellite results were mostly concordant with the mtDNA phylogenetic reconstruction, with exception of one sample (H176_NE) that clusters with *P. a. auritus* on mtDNA but clearly belongs to the lineage *P. a. begognae* when considering the

microsatellites results (Fig. 2.1a). Comparison of microsatellites' results with the nDNA concatenated reconstruction (Fig. 2.1b), evidenced two samples (H170_NE and H175_NE) classified within the *P. a. auritus* microsatellite group that are included within the *P. a. auritus/P. a. begognae* phylogenetic clade.

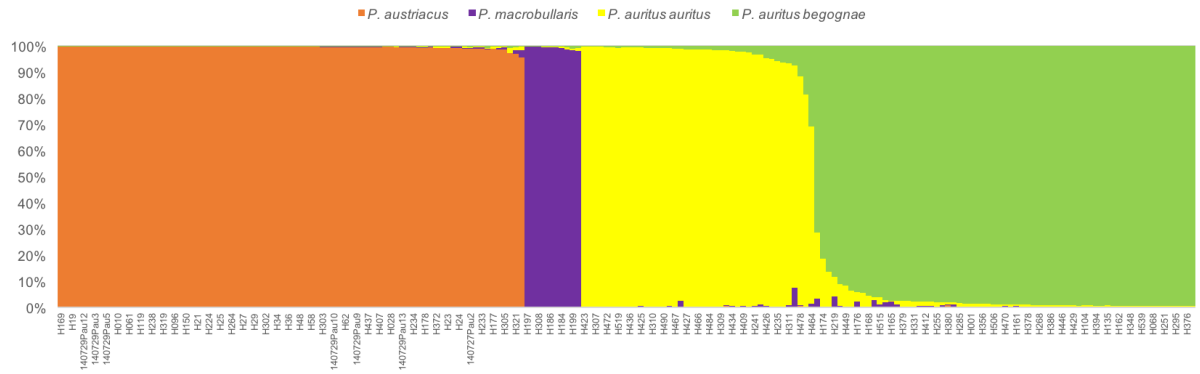

Figure 2.3 - STRUCTURE bar plot illustrating the genetic structure of the four populations (K=4), *P. austriacus* in orange, *P. macrobullaris* in purple, *P. a. auritus* in yellow and *P. a. begognae* in green. Coloured bars represent the probability of assignment of each individual to a cluster.

DISCUSSION

The use of phylogenetic and microsatellite analyses allowed for better insights on the evolutionary history of the lineages of the genus *Plecotus* in the Iberian Peninsula. Both phylogenetic analyses, mitochondrial and nuclear, and microsatellite's structure showed a clear separation of the lineages *austriacus* and *macrobullaris*, supporting current taxonomic classification (Corbet, 1978; Spitzenberger *et al*., 2003). Phylogenetic reconstructions of the nuclear introns showed to be unable to differentiate between the lineages *a. auritus* and *a. begognae*, however, the mitochondrial reconstruction separated these lineages, with a high level of differentiation. The microsatellite analyses also showed a clear differentiation between these lineages, with limited admixture (gene flow).

Species of the genus *Plecotus* are known to have high morphological similarities, making them hard to identify in the field by simple morphological characteristics alone (Spitzenberger *et al.*, 2001; Mucedda *et al.*, 2002; Juste *et al.*, 2004; Ashrafi *et al.*, 2010). This arises difficulties when collecting samples for these species, as misidentifications can frequently occur. In our study, we came across several incongruences in field species identifications, even in well described species such as *P. austriacus* and *P. auritus*. In fact, Spitzenberger *et al.* (2006)

found geographic variations in the coloration and skull measurements expressed in several specimens of *P. austriacus*, while genetically identical. This supports the fact that morphological characterisation by itself may be misleading when used to identify closely related species, and it is imperative to ensue molecular identification as well. Additionally, some samples were not incorporated on the nDNA concatenated tree because they did not amplify for a variety of fragments. This might result likely from low DNA quality for reactions more sensible to it, but can also result from mutations in the flanking regions where the primers should anneal.

Differences revealed in the mtDNA reconstruction could be due to phenomena such as male-biased dispersal and female philopatry, which has shown to be common in several bat species (McCracken & Wilkinson, 2000; Burland & Wilmer, 2001; Ruedi & Castella, 2003; Rivers *et al.*, 2005; Senior *et al.*, 2005; Safi *et al.*, 2007; Ibáñez *et al.*, 2009; Rodrigues *et al.*, 2010). Female philopatry is known to provoke higher genetic differentiation between bat colonies (Moussy *et al.*, 2013). Several studies have in fact found high levels of philopatry in *Plecotus* species in Europe (Entwistle *et al.*, 2000; Furmankiewicz & Altringham, 2007). However, by sampling individuals outside of colonies and complementing the analyses with microsatellites, which demonstrate high levels of separation between the Iberian samples of *P. a. auritus*, we believe that the results show enough support to suggest the existence of the Iberian lineage. In fact, several subspecies of *Plecotus* spp. have been found through mitochondrial differences. Before being recognised as a species (Kiefer *et al.*, 2002), *P. kolombatovici* was found as a subspecies of *P. austriacus*, *P. a. kolombatovici* (Mayer & von Helversen, 2001). Also, Spitzenberger *et al.* (2006) found evidence of a subspecies of *P. kolombatovici* in Cyrenaica, *P. k. gaisleri*. Mitochondrial fragments have also allowed the taxonomical clarification of other species of the genus *Plecotus*, such as in the case of Spitzenberger *et al.* (2003), which found that *P. alpinus* and *P. microdontus* were in fact *P. macrobullaris*. Moreover, the combination of mitochondrial and nuclear fragments in phylogenetic analyses has allowed the confirmation of several long-eared bat subspecies in North America (Piaggio & Perkins, 2005). The existence of intraspecific differentiation is fairly common in bats, since a considerable amount of subspecies have also been found in other genus, such as *Myotis*, *Eptesicus*, *Miniopterus*, *Barbastella* and *Rhinolophus* (Ruedi & Mayer, 2001; Juste *et al.*, 2003; Jones *et al.*, 2006; Sun *et al.*, 2009; Šrámek *et al.*, 2012; Juste *et al.*, 2013; Puechmaille *et al.*, 2014).

In order to clarify the existence of possible hidden evolutionary significant units within taxa, and further clarify species evolutionary history and phylogenetic structure, particularly in bats,

we encourage the employment of multiple molecular markers (nDNA, mtDNA and microsattelites), to ensure a better evolutionary resolution and to ensure species are not taxonomically inflated (Godinho *et al.*, 2008; Zachos *et al.*, 2013).

Evolutionary history of *Plecotus auritus* in Iberia

Mitochondrial and microsatellite analyses provide strong support for the divergence between the lineages *P. a. auritus* and *P. a. begognae* into distinct clades, and therefore, revealing *P. a. begognae* as a different evolutionary unit. It would seem that a possible isolation between the Iberian and the European populations of *P. a. auritus*, left a particular genetic structure in *P. a. begognae*, corresponding to patterns of lineage divergence, which could be reflected in a regional endemism (e. g. Igea *et al.*, 2013; Razgour, 2015). Although the Iberian Peninsula was the main source of post-glacial recolonization for several bat species (Ruedi & Castella, 2003; Rossiter *et al.*, 2007; Razgour *et al.*, 2013), it would seem that Iberian populations of *P. a. auritus*, never expanded their range beyond the peninsula, remaining isolated in Iberia and differentiating into the lineage *P. a. begognae*, following the same pattern of *Barbastella barbastellus* (a forest dwelling bat member of the Plecotinii sub-family), whose Iberian populations did not had a relevant contribution in European post-glacial expansion (Rebelo *et al*., 2012). This is reflected in current *P. a. auritus* and *P. a. begognae*'s distributional range, with *P. a. begognae* remaining in the northern regions within Iberia, while *P. a. auritus* is restricted to the Pyrenees mountain range, showing parapatric distribution patterns (Santos *et al.*, 2014).

Our results showed that phylogenetic reconstructions based of nuclear introns may not be sufficient to differentiate lineage's evolutionary structure, as they did not present enough resolution to separate *P. a. auritus* from *P. a. begognae*. This is probably due to the slow evolutionary rate presented by nuclear fragments, which are known to be only sensitive to longer periods of isolation between lineages (Wan *et al.*, 2004). In our case, they have proven ineffective in the identification of different evolutionary units, which is a key interest, especially in the study of cryptic species.

Such is not the case for mitochondrial fragments, where a clear lineage separation between *P. a. auritus* and *P. a. begognae* can be observed. This separation has been previously demonstrated with other mtDNA fragments, such as in the cytochrome b (cyt b), NADH dehydrogenase gene 1 (ND1), 16S rRNA (16S) and the control region (CR) (Kiefer *et al.*, 2002; Juste *et al.*, 2004; Ibáñez *et al.*, 2006; Spitzenberger *et al.*, 2006). This is due to the

mtDNA rapid evolutionary rate, facilitating the identification of recently evolved taxa, mostly outperforming nDNA phylogenetic reconstructions (Moore, 1995). However, its use may be limited, because mitochondrial genes are maternally inherited as a single linkage group (haplotype) and provide only one independent estimate of the species tree. As such, it is advised to also analyse bi-parentally inherited nuclear markers with different evolutionary rates (Razgour *et al.*, 2015).

By adding microsatellite analyses, we were able to further inform on the evolutionary structure between *P. a. auritus* and *P. a. begognae*, while successfully identifying *P. a. begognae* as a distinct evolutionary unit. Microsatellites have a higher evolutionary rate, being able to detect differences on the species' population level (Wan *et al.*, 2004). Our microsatellite results support the separation of the lineage *P. a. begognae*, even though some level of admixture and gene flow can be observed, which is highly likely in the contact zone located in the Pyrenees (Santos *et al.*, 2014). However, it is not uncommon to observe the presence of gene flow and occasional hybridization in closely related lineages (Eckert & Carstens, 2008). These results demonstrate that, after a possible isolation between the European and the Iberian populations of *P. a. auritus*, the two lineages are showing some recent level of admixture.

The different results obtained by the distinct molecular markers provide support that *P. a. begognae* thrived in the Iberian Peninsula during the last glacial period, although isolated from their European counterparts – this is shown by the mtDNA differentiation. During the post-glacial colonization of Europe, *P. a. begognae* seemed to have colonized all suitable habitats in Iberia but we have no evidence that this bat has passed the Pyrenees range. A possible hypothesis for this is that the European populations of *P. a. auritus* has probably reached the Pyrenees first and hampered *P. a. begognae*'s expansion. Therefore, the evolutionary history of *P. a. begognae* could be summarized by long periods of isolation from their European counterparts during the Quaternary glacial cycles (which on average last ca. 100 000 years; O'Regan, 2008), followed by short periods of limited admixture (gene flow) during the interglacial cycles (lasting on average between 10 000 to 20 000 years), where contact zones may be established, at the margin of the Iberian Peninsula, i.e., the Pyrenees.

Consequently, even with the lack of confirmation by nDNA reconstructions, and supporting solely in mtDNA and microsatellite analyses, we are able to distinguish *P. a. begognae* as a different lineage and evolutionary significant unit. These results allow us to support the taxonomic status of *P. a. begognae* as an Iberian subspecies of *P. a. auritus*.

Future work

While our results seem to support the subspecies status for *P. a. begognae*, further analyses can complement relevant information for the taxon. It would be of interest to observe results with a more numerous dataset, i.e. more samples of *P. a. begognae* and *P. a. auritus* from the Iberian Peninsula, and with different nuclear and mitochondrial markers. Furthermore, analyses on mitogenomes could also present valuable evolutionary information (Botero-Castro *et al.*, 2013; Mata *et al.*, 2017) on *Plecotus* spp.

Since these two taxa seem to present a parapatric distribution in Iberia (Santos *et al.*, 2014), with *P. a. auritus* mainly restricted to the Pyrenees and *P. a. begognae* occurring throughout the rest of northern Iberia, phylogeographic studies would also be of interest as they would complement our phylogenetic approach, and help in understanding if biogeographic patterns are related to genetic ones, as well as identifying the location of the ancestral populations and vicariant episodes. Moreover, with a larger sample size, additional studies on male dispersal and female philopatry in these *Plecotus* species could provide more evidence on their population structure and further understand the potential speciation process that is ongoing. Complementarily, hybridization studies could also help in better understanding the patterns of gene flow occurring between *P. a. auritus* and *P. a. begognae*.

Additionally, morphology analyses would be of high relevance in order to understand if there are significant morphological differences in the Iberian subspecies. de Paz (1994) stated that *P. a. begognae* had greater cranial dimensions than *P. a. auritus*, with the exception the breadths of the tympanic bulla and interorbital constriction. Also, with the exception of the hind foot, thumb and tragus, external dimensions were described as greater than *P. a. auritus*, presenting brown dorsum and slightly paler brown venter, with basally chocolate brown hairs. The tragus was presented as translucent brown with a slight pigmentation as in *P. a. auritus*, and measurements of the bacula did not present significant differences. This could have been due to the low sampling size or lack of genetic characterisation to differentiate the lineages. Studies in the morphology of species of the genus *Plecotus* have shown that commonly used measurement indices are insufficient to differentiate species of this genus and a more intricate analysis is necessary (Ashrafi *et al.*, 2010), particularly of the bacula, which have shown to be a diagnosing feature in species of this genus (Mucedda *et al.*, 2002; Spitzenberger *et al.*, 2006; Ashrafi *et al.*, 2010; Herdina *et al.*, 2010). As such, an increased dataset of *P. a. begognae* and *P. a. auritus* from the Iberian Peninsula is needed to further clarify these measurements and complement them with molecular analyses.

The recognition of this lineage as a subspecies, not only calls for further molecular studies, but also for a deeper understanding of species ecological and conservation requirements. Moreover, evidence of bat cryptic diversity in the Iberian Peninsula makes it an area of high relevance for molecular and ecological studies and the presence of other poorly studied cryptic species calls for further research.

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SUPPORTING INFORMATION II

Appendix S1 – Study area

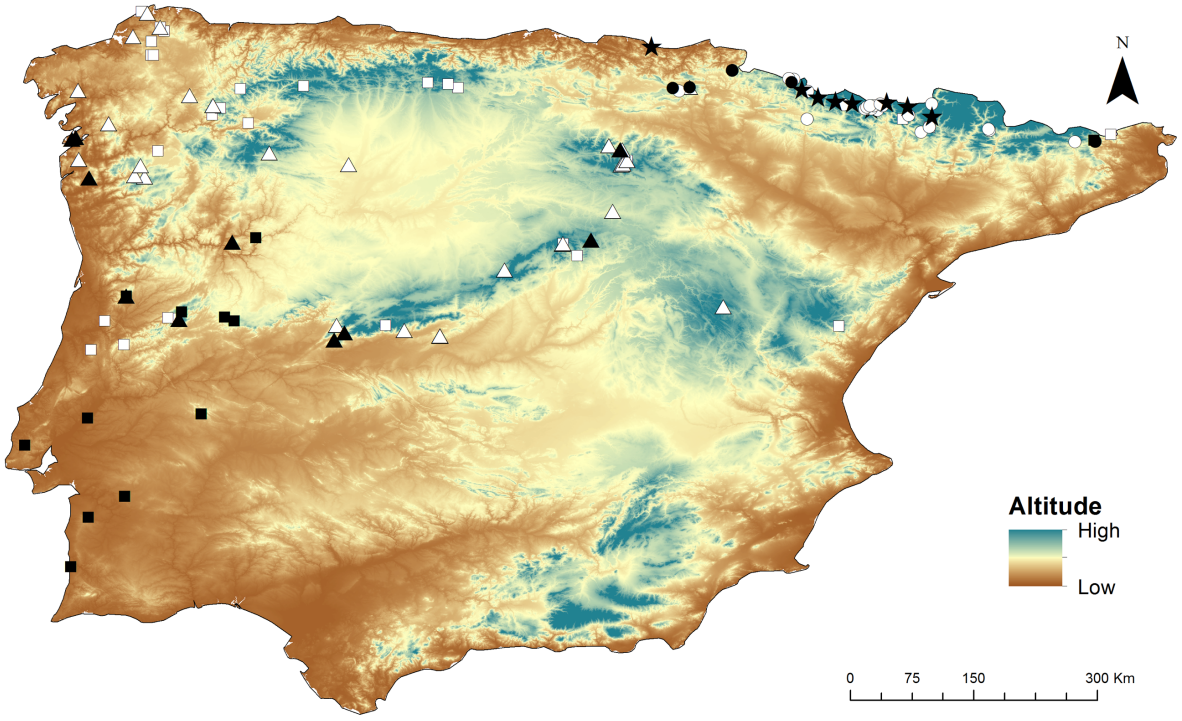

Fig. S1.1 – Samples used in this study with topography in the Iberian Peninsula. *Plecotus austriacus* are represented by squares, *Plecotus macrobullaris* by stars, *Plecotus auritus auritus* by circles and *Plecotus auritus begognae* by triangles. Black symbols represent the samples used in the phylogenetic study and white symbols the samples used in the subsequent microsatellite analyses. Species assignment was performed with microsatellite analyses with qi>0.95 (see Fig. S3.3, Appendix S3).

Appendix S2 – PCR and thermocycling conditions

For the five fragments used in this work, amplifications were performed including 0.1 units of MyTAQ Mix (Bioline) following manufacturer's instructions, 0.5 μM of each primer and approximately 10 ng of genomic DNA in a total volume of 10 μl. Thermocycling conditions were as shown in Table S2.1. Denaturation and extension temperatures were 95°C and 72°C, respectively, for 30 seconds.

Table S2.1. Thermocycling conditions for the mtDNA and four nuclear fragments amplified in this work.

Genomic compartment	Fragments	Annealing temperature	Extension time	N cycles
mtDNA	COI	58°C down 0.5°C per cycle to 56º	30 sec	37
nDNA	ABHD11, ACOX2	65°C down 0.5°C per cycle to 63º	30 sec	37
nDNA	ACPT, ROGDI	63°C	30 sec	40

Microsatellite loci were amplified in three multiplex reactions according to Table S2.2. Total reaction volume was 10 ul, including 5 ul of the QIAGEN PCR Master Mix, 1 ul of primer mix, and 2 ul of DNA. Forward primers were M13-tailed to follow a fluorescent labelling protocol (Blacket *et al*., 2002). A negative control was always included in all reaction sets to monitor possible contaminants. PCR Thermo-cycling conditions are described in Figure S2.1.

Table S2.2 – Microsatellite loci genotyped in our study. Three multiplex panels were used using four dyes. Allele ranges and references for microsatellite development are given.

Loci name	Multiplex	Dye	Allele range	Reference
Paus20	1	FAM	98-122	Razgour *et al*. 2013
Paus15	1	NED	101-134	Razgour *et al*. 2013
Paur06	1	PET	143-169	Burland *et al*. 1998
Paus17	1	FAM	162-185	Razgour *et al*. 2013
Paus11	1	VIC	175-187	Razgour *et al*. 2013
Paus19	1	NED	184-226	Razgour *et al*. 2013
Paus03	1	FAM	246-257	Razgour *et al*. 2013
Paus04	1	VIC	256-309	Razgour *et al*. 2013
Paus06	2	VIC	131-158	Razgour *et al*. 2013
Paus18	2	FAM	134-157	Razgour *et al*. 2013
Paus14	2	NED	145-181	Razgour *et al*. 2013
Paus08	2	PET	147-171	Razgour *et al*. 2013
Paus13	2	FAM	172-199	Razgour *et al*. 2013
Paus07	2	VIC	212-245	Razgour *et al*. 2013
Paur05	2	FAM	229-249	Burland *et al*. 1998
Paus09	2	NED	260-262	Razgour *et al*. 2013
Paus10	3	VIC	94-135	Razgour *et al*. 2013
Paur01	3	NED	132-159	Burland *et al*. 1998
Paus16	3	PET	133-187	Razgour *et al*. 2013
Paus12	3	FAM	134-174	Razgour *et al*. 2013
Paus01	3	VIC	184-208	Razgour *et al*. 2013
Paus02	3	NED	185-214	Razgour *et al*. 2013
Paus05	3	FAM	234-263	Razgour *et al*. 2013

Steps	Temperature	Time	Cycles
1	95°C	15 min	
2	95°C 58°C down 0.5°C per cycle 72°C	3 sec 1 min 45 sec	13
3	95°C 52°C 72°C	30 sec 1 min 45 sec	27
4	60°C	30 min	

Figure S2.1 – Thermocycling conditions used to amplify loci included in multiplexes 1, 2 and 3 described in Table S2.2.

Appendix S3 – Population genetic structure

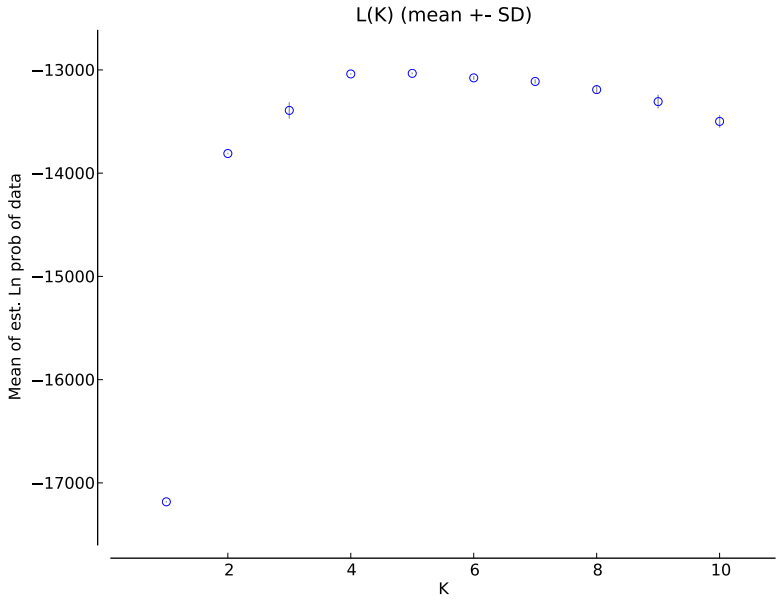

Figure S3.1 – Plot of mean likelihood L(K) and variance per K value from STRUCTURE on a dataset containing 200 individuals genotyped for 18 polymorphic microsatellites.

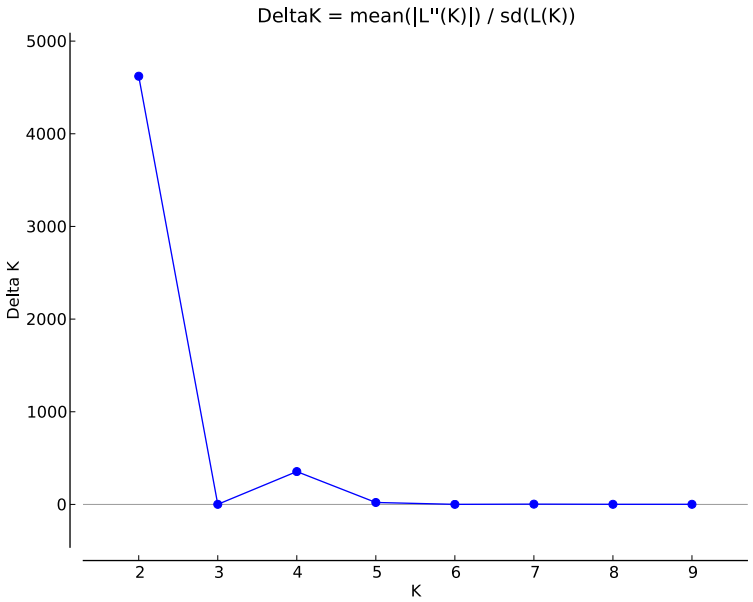

Figure S3.2 – Evanno *et al.* (2005) plot for detecting the number of K groups that best fit the data (data as in Fig. S3.1).

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CHAPTER 4

LANDSCAPE GENETICS AND CONNECTIVITY OF *PLECOTUS AURITUS BEGOGNAE*

PAPER III - GO WITH THE FLOW! INTEGRATING LANDSCAPE GENETICS WITH CONNECTIVITY ANALYSES TO DESIGN SPATIAL EXPLICIT CONSERVATION

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Paper in preparation.

ABSTRACT

Landscape composition plays a significant role in the shaping of species distributions, population structure and connectivity. Currently, one of the main methodological challenges is the integration of these methodologies into a single analytical approach. Landscape genetics tests the effect of landscape and environmental variables on the spatial genetic structure of populations. While this approach allows to identify the main drivers and barriers to gene flow, it does not consider the connectivity between populations. Thus, areas susceptible to isolation may be overlooked. In here, we use a multi-disciplinary approach to identify the main environmental drivers of the population structure and connectivity of *Plecotus auritus begognae* in the Iberian Peninsula. We have gathered over 370 samples, covering the whole Iberian range of the species, and genotyped those samples for 19 microsatellite loci. For the spatial analyses, we considered several environmental variables divided into three sets: land cover, topography and climatic. Our results show that there was no spatial structure of the populations within the bat range in Iberia (K=1 populations), i.e. high levels of gene flow were detected. Yet, the landscape variables distance to forests and to slopes seemed to contribute to the maintenance of current gene flow patterns. The large extents of forests in the northern and central regions of Iberia seem to prevent the fragmentation of the populations of *P. a. begognae*. Nonetheless, the connectivity analyses showed that areas in the north-west and centre-east of Iberia are susceptible of becoming isolated because their connectivity with the bat's core distribution is dependent on the occurrence of small forests in between. In conclusion, the integration of landscape genetics with connectivity analyses has provided spatially explicit results that have identified areas sensitive to genetic isolation. This information can also be used to develop tailor-suited conservation measures for the species.

INTRODUCTION

The establishment of high priority areas for conservation and protected areas has been the main focus of many policy and governmental directives (Deguignet *et al.*, 2014). In the last years, several methods were developed to assist and support on the design of conservation measures, particularly spatial prioritization of areas for conservation, such as establishing biodiversity hotspots and key habitat areas (Myers *et al.*, 2000; Eken *et al.*, 2004). Several tools are now available to aid in the identification of important areas for the persistence of nature and techniques have advanced beyond merely representing natural features to including species distribution and climate change modelling (Araújo *et al.*, 2011; Lyet *et al.*,

2013), population analyses (Guisan & Thuiller, 2005; Torres *et al.*, 2012) and accounting for uncertainty and economic costs (Gantioler *et al.*, 2014; Vicente *et al.*, 2016).

Spatially-explicit methods, like ecological niche modelling and systematic conservation planning, allowed us to identify priority areas by using single and multi-species approaches along with landscape features that are known to influence species' distribution patterns. These methods have proven successful in a number of conservation applications, such as conservation planning and delimitation of protected areas (Araújo & Williams, 2000; Ferrier *et al.*, 2007), aiding in field survey planning for rare or undiscovered species and distribution areas (Engler *et al.*, 2004; Pearson *et al.*, 2007), assessing potential impacts of climate change (Araújo *et al.*, 2004; Thomas *et al.*, 2004), evaluating species invasions (Beerling *et al.*, 1995; Peterson, 2003; Vicente *et al.*, 2016), testing biogeographical, ecological and evolutionary hypotheses (Anderson *et al.*, 2002; Graham *et al.*, 2004) and quantifying the environmental niche of species (Austin *et al.*, 1990; Vetaas, 2002). However, spatial approaches do not take into account the genetic characteristics of and between species, such as population structure, genetic variability, gene flow and phylogenetic relationships, whose knowledge is essential for identifying which habitats or parts of the range of species are in need of conservations actions (Eckert *et al.*, 2008).

On the other hand, population genetic studies allow us to identify several hard-to-observe processes that can be applicable to species conservation management, such as identifying evolutionarily significant units and management units, which allow us to understand which taxa or populations should be prioritized, calculating historical effective population size, assessing sex-biased dispersal, identifying population bottlenecks, and characterizing population dynamics. Nevertheless, molecular methods tend to overlook spatial the contribution of the landscape on the genetic structure of species and its role on the maintenance of genetic variability (Galpern *et al.*, 2012).

The recent field of landscape genetics offers an interdisciplinary framework for combining tools and concepts from both landscape ecology and population genetics, relating the effects of landscape structure on the movement of organisms to patterns of genetic variation. It combines landscape ecological methods, spatial statistics and population genetic analyses (Holderegger & Wagner, 2008), allowing the identification of which geographical and environmental features of the landscape, i.e. biogeographical dispersal barriers and corridors, are influencing genetic variation and gene flow. However, despite its promising outputs, the practical applications of studies on landscape genetics have been seldom used by

practitioners or applied for conservation and management purposes (Keller *et al.*, 2015). Moreover, the application of landscape genetics to infer connectivity between populations frequently lacks a quantitative approach to evaluate which habitats or parts of the species distributions should be prioritized for conservation (Storfer *et al.*, 2007; Galpern *et al.*, 2012). This constitutes a major pitfall since connectivity has been recently highlighted of utmost importance in conservation, as it allows the understanding of how fragmented are species' habitats, evaluating which areas of a species distribution are reachable and connected to remaining species range (Ament *et al.*, 2014). The restriction of dispersal and gene flow promotes the loss of genetic variation through genetic drift, especially for small or isolated populations, or for species with limited dispersal abilities (Galpern *et al.*, 2012).

Connectivity can be defined as the movement of individuals and genes among resource patches, relating to dispersal across landscapes (Vasudev *et al.*, 2015). These linkages are known to influence population structure and dynamics, consequently, species' distribution patterns, population demographics, genetic variability, evolutionary processes and more. Moreover, connectivity is also increasingly relevant for conservation, since its loss is related to habitat fragmentation, one of the major causes of decline or extinction of species as a result of human pressure (Tournant *et al.*, 2013). Reduced landscape connectivity not only interferes with dispersal, gene flow and wildlife migration and breeding, but can result in small, isolated populations, which have increasing risk of extinction related to a series of processes like inbreeding depression, demographic stochasticity and reduced opportunity for rescue (Koen *et al.*, 2014). Improving landscape connectivity is a key strategy of recent biodiversity conservation policies, and is considered one of the best responses to counter the adverse effects of habitat fragmentation and facilitate the shifts in the natural ranges of species due to climate change (Saura & Pascual-Hortal, 2007; Saura *et al.*, 2011; Saura & de la Fuente, 2017). It is therefore essential to consider connectivity as a basis for conservation planning and landscape change analysis.

In this study, we propose a multidisciplinary approach to integrate landscape genetics with spatially explicit landscape connectivity as a more precise tool for the prioritization of areas for conservation. On one hand, landscape genetics allows us to identify the genetic variation of populations as well as gene flow patterns and which landscape and environmental features are affecting them, and on the other hand, landscape connectivity analysis evaluates how connected are these populations. By merging the two disciplines, we are able to better understand the genetic and movement dynamics of the populations while also identifying the

source populations and populations at risk, and which habitats or areas should be priority for conservation.

Bats constitute a globally distributed and highly diverse taxonomic group of great conservation value. However, bats also have volant dispersal capabilities that allow them to cross biogeographical barriers making them challenging to study in landscape genetics. Still, bat species also exhibit high diversity in population structure patterns mostly deriving from differential movement capabilities, migration dynamics, roosting and mating behaviours and social organization (Kerth *et al.*, 2000; Russo *et al.*, 2005; Jones *et al.*, 2009; Russo *et al.*, 2016). Such is the case of the Iberian subspecies of the Brown Long-eared Bat, *Plecotus auritus*, hereafter *Plecotus auritus begognae*. This subspecies was used as a case study to apply our approach. In previous work, our team has identified *P. a. begognae* as a subspecies of the European *P. auritus*, hereafter *Plecotus auritus auritus*, and determined its distribution range and ecological requirements. Moreover, this species is known to have limited dispersal capability and, consequently, prominent population subdivision (Burland *et al.*, 1999), suggesting it could be strongly affected by landscape and environmental features that could present as barriers to dispersal and gene flow, making it a promising subject for our research.

In this study, we aimed to identify which areas within the Iberian range are priority for the conservation of *P. a. begognae*. Specifically, we aimed to answer the following questions:
1. How are Iberian populations genetically structured?
2. Which landscape elements and/or climatic conditions promote gene flow between Iberian populations?
3. Where are the main gene flow routes located in Iberia?
4. Which regions in Iberia are key for maintenance of population connectivity/gene flow?
In a novel approach, we combined molecular techniques with species distribution modelling, landscape genetics and connectivity analyses to develop a multidisciplinary methodology, to ultimately identify the priority habitat regions in Iberia for the preservation of *P. a. begognae*'s populations and maintenance of gene flow.

MATERIALS AND METHODS

Study area

The study area was the Iberian Peninsula, located in Europe's south-western extremity. It covers nearly 600 000 km^2 and it is bordered to the south and east by the Mediterranean Sea and to the north and west by the Atlantic Ocean, being divided from the remainder of Europe by the Pyrenees mountain range in the north-east. It has a very heterogeneous topography and is roughly characterized by two main biogeographical regions: Eurosiberian and Mediterranean (Sillero *et al.*, 2009; Romo & García-Barros, 2010). The study area was restricted using a 100km buffer south of the southernmost location for the bat species (Santos *et al.*, 2014) (Fig. 3.1), avoiding the inclusion of unnecessary background data that could overfit the spatial models (Merow *et al.*, 2013).

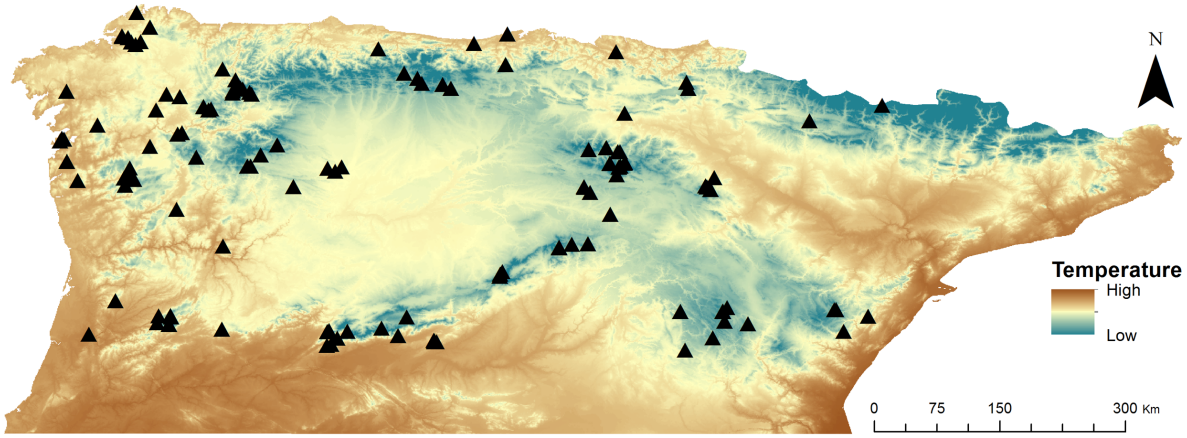

Figure 3.1 - Mean annual temperature of the study area and *Plecotus auritus begognae* records (black triangles). Each symbol may correspond to more than one location.

Sample collection

A total of 373 samples of *Plecotus a. auritus* and *Plecotus a. begognae*, were obtained from mist netting and roost trapping sessions throughout the Iberian Peninsula (Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Rebelo *et al.*, 2012; Santos *et al.*, 2014), spanning the entire known range of the subspecies *P. a. begognae*. For each specimen, a tissue sample was collected in the field, through a small biopsy punch in the wing membrane and all samples were stored in ethanol for future laboratory analyses. Species identification

for all records was validated by molecular analyses, following the procedure described by Ibáñez *et al.* (2006) and used previously in Santos *et al.* (2014).

Multiple approach methodology

To prioritize and identify regions for conservation, several techniques were employed in this study, here described in numerical steps and represented in an analytical framework (Fig. 3.2).

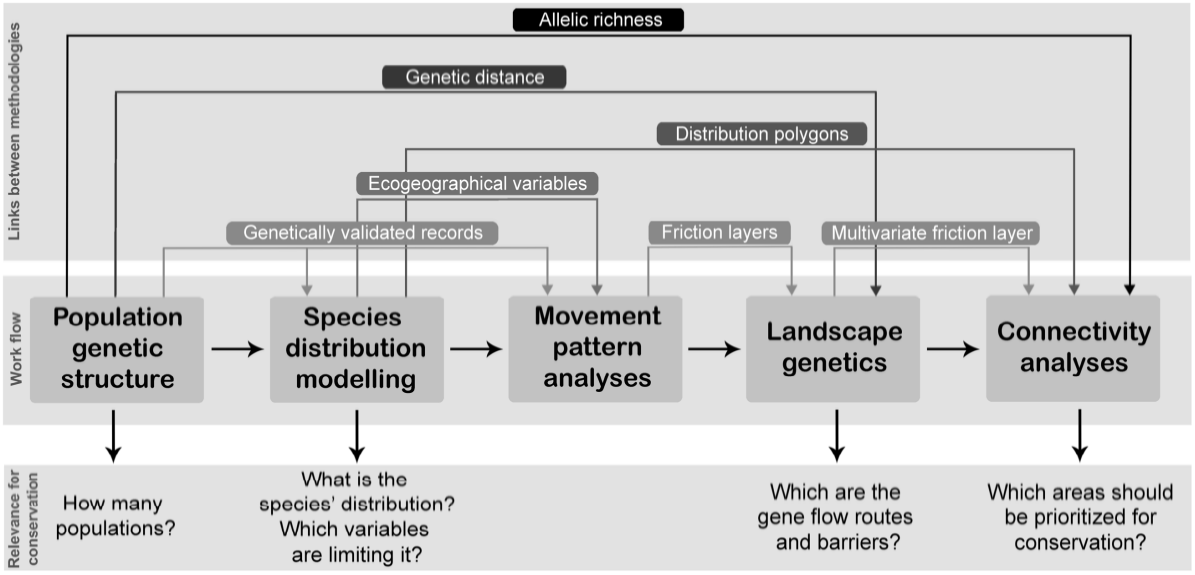

Figure 3.2 - Flow chart representing the multiple methodologies used.

1. Genetic analyses

Genomic DNA was extracted from the whole wing membrane punch using the QIAamp DNA Micro Kit (QIAGEN). Individual genotyping of 23 autosomal microsatellite loci previously developed for *P. austriacus* (n=20; Razgour *et al.*, 2013) and *P. auritus* (n=3; Burland *et al.*, 1998) was performed using three multiplex reactions (Appendix S1, Supporting information III, outlines markers per multiplex panel, allele range, PCR reaction conditions and PCR cycle programs). Forward primers were M13-tailed to follow a fluorescent labelling protocol (Blacket *et al.*, 2012). A negative control was always used to monitor possible contaminants. PCR products were separated by size in an ABI3130xl genetic analyser. Alleles were scored against the GeneScan500 LIZ size standard using GENEMAPPER 4.0 (Applied Biosystems) and checked manually by two observers. The accuracy of the results was measured through

re-amplification of 15% random selected samples for each locus (Bonin *et al.*, 2004) resulting in complete concordance among replicates. Four loci exhibiting low amplification rates were removed from analysis.

As a first approach to look for genetic population structure, we conducted a Bayesian clustering analysis implemented in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000; Falush *et al.*, 2007) with all genotyped samples, including both *P. a. auritus* and *P. a. begognae*, and allowing K=1 to K=10. Individual membership proportions (qi) were assessed using the admixture model in 10 independent runs each with 10^6 MCMC iterations following a burn-in period of 10^5 iterations and no prior population information. The threshold level to differentiate *P. a. auritus* and *P. a. begognae* with our set of microsatellites was defined based on the power of STRUCTURE to assign simulated individuals with prior known ancestry. We used 40 *P. a. auritus* and *P. a. begognae* samples that exhibited qi>0.90 on the previous STRUCTURE run (K=2) to generate 100 simulated genotypes for each lineage using HYBRIDLAB 1.0 (Nielsen *et al.*, 2006). Simulated genotypes were run in STRUCTURE using the admixture model and no prior population information. The minimum value of individual assignment of simulated genotypes from *P. a. auritus* and *P. a. begognae* was further used as the threshold to assign individual results of the first STRUCTURE to each lineage, or otherwise to classify individuals as admixed. A second STRUCTURE analysis with the same settings of the first, was then ran using only individuals classified as *P. a. begognae*.

Multilocus genotypes of *P. a. begognae* and *P.a. auritus* were used to estimate nuclear diversity based on the number of alleles per locus (NA), and observed (H_O) and expected (H_E) heterozygosity for each locus using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). Homozygosity by loci (HL) of each sample was calculated using Cernicalin V.1 (Aparicio *et al.*, 2006). ARLEQUIN was also used to estimate departures from Hardy–Weinberg equilibrium (HWE) following (Guo & Thompson, 1992) and to evaluate pairwise linkage disequilibrium. Statistical significance was adjusted using sequential Bonferroni corrections. Finally, we calculated a pairwise individual genotypic distance matrix between *P. a. begognae* samples using GenAlEx 6.5 (Smouse & Peakall, 1999; Peakall & Smouse, 2012). A geographic distance matrix between these samples was also calculated, through the packages “maps” (Becker & Wilks, 2017) and “reshape2” (Wickham, 2007) in R version 3.3.2 (R Core Team, 2012).

2. Species distribution modelling

The ecogeographical variables used for species distribution modelling in this study were based on (Santos *et al.*, 2014), where the most relevant variables for cryptic bat species' occurrence in the Iberian Peninsula were based on an initial set of 45 variables. The number of variables was then decreased by first eliminating the highly correlated variables and then considering only the most relevant ones, by using the percentage contribution values and the jackknife values of regularized training gain (Elith *et al.*, 2011), obtained in the species distribution models for each species. Landscape genetics requires the standardization of spatial variables (Spear *et al.*, 2010; Koen *et al.*, 2012), hence to have comparable results from species distribution models, all variables were standardized prior to spatial analyses. The final set of 15 ecogeographical variables (EGVs) (see Table S2.1, Appendix S2 in Supporting Information) had a resolution of approximately 1×1 km and were standardized through the formula [("raster" - "raster".minimum) / ("raster".maximum - "raster".minimum) x 100]. All respective calculations were made in ArcGIS 10.1 (ESRI, 2012).

Species distribution models (SDMs) were calculated using the maximum entropy modelling technique, Maxent 3.3.3k (Phillips *et al.*, 2006), which has shown to be a powerful algorithm when dealing with limited datasets, particularly in bats (Rebelo & Jones, 2010; Santos *et al.*, 2014; Razgour *et al.*, 2016). Initially, spatial autocorrelation within the records was eliminated using the spatially rarefy occurrence data tool in SDMtoolbox (Brown, 2014). These presence records, as the dependent variables, and the selected 15 EGVs (Table S2.1, Appendix S2), as the independent variables, were then imported into Maxent and run in auto features, with 20 cross-validation model replicates (Merow *et al.*, 2013). The resulting most relevant variables, were then used to run univariate SDMs in order to obtain continuous resistance surfaces for connectivity analyses. The full model was then reran using the same characteristics as the univariate models, which were ran in auto features, with cumulative output format and no replicates.

3. Movement pattern analyses

For the movement pattern analyses, we considered the set of univariate models of the most important variables, the SDM and a univariate model of the climate variable mean autumn temperature, the latter was deemed important for species' dispersal and connectivity (Razgour *et al.*, 2014). In order to avoid subjectivity of arbitrary cost values (Koen *et al.*, 2012), we decided to use the univariate SMDs calculated as input continuous resistance surfaces. These

resistance surfaces were then used to calculate friction layers in Circuitscape 4.0.5 (McRae, 2006; McRae *et al.*, 2008). Circuitscape is a connectivity analysis software, which borrows algorithms from electronic circuit theory to predict patterns of movement, gene flow, and genetic differentiation among populations in heterogeneous landscapes. Circuit theory complements least-cost path approaches because it considers effects of all possible pathways across a landscape simultaneously (McRae & Beier, 2007). Since Circuitscape requires surfaces represented as conductive or resistance, all our input variables were scaled from 1 to 101 and then assigned as conductance surfaces, where lower values, i.e. lower probability of species' occurrence, were assigned to movement barriers and higher values, i.e. higher probability of species' occurrence, were assigned to features which promote gene flow. Landscape resistance matrices of the samples were then built for each of the variables, along with univariate friction layers.

4. Landscape genetics

In order to determine which ecogeographical variables were affecting gene flow of *P. a. begognae* in Iberia, multiple regressions on distance matrices (MRDMs) (Legendre *et al.*, 1994) were calculated, using the resulting landscape resistance matrices from Circuitscape, along with genetic and geographic distance matrices previously described in the genetic analyses section. MRDMs have demonstrated to be highly accurate when compared with other regression statistical methods, and have shown to powerfully balance type 1 errors (Balkenhol *et al.*, 2009; Roffler *et al.*, 2016). All calculations were performed in R version 3.3.2 (R Core Team, 2012).

In a first approach, all distance matrices were imported into R and standardized from 0 to 1, using the "scales" package (Wickham, 2016). Secondly, geographic variation was removed from all of the variables and the genetic distance, by calculating the residuals of linear models of geographic distance against each of the landscape variables (Dyer *et al.*, 2010). This step was performed because the effect of geographic distance, which is known to affect populations' genetic structure (Razgour *et al.*, 2014), could mask the role that landscape elements and geographical barriers may play in structuring patterns of gene flow.

In order to understand the effect of the landscape variables on genetic divergence, the "ecodist" (Goslee & Urban, 2007) package was used to run MRDMs. First, univariate MRDMs of genetic distance were calculated for each of the variables, in order to identify which had significant correlations with genetic differentiation. After, using the significant variables

($p<0.05$) obtained in the univariate models, multivariate MRDMs of genetic distance were calculated, determining which were the final drivers of gene flow and their respective weights in the final model.

The resulting final model identified the most significant ecogeographical variables related with genetic divergence and their estimates, allowing us to spatially project this model to build a multivariate friction layer. Using the friction layers of each of the significant variables obtained in Circuitscape, along with their estimate values in the final MRDM model, we multiplied each of the variable's estimate values with their respective friction layer and added them in order to obtain a final resistance surface.

5. Connectivity analyses

After establishing the main routes of *P. a. begognae*'s gene flow in Iberia and their drivers through the landscape genetics analyses, we aimed to determine which distribution patches were more relevant for the maintenance of connectivity and gene flow.

Primarily, we converted the previously calculated SDM into habitat patches of species occurrence within the study area. The SDM was reclassified into presence-absence using the maximum training sensitivity plus specificity logistic threshold value (Liu *et al.*, 2013), and then converted into polygons (hereafter patches) using ArcGIS 10.1 (ESRI, 2012). Only the polygons that had *P. a. begognae* samples were selected resulting in that less than 31% of the total predicted habitat suitability area for the subspecies was not covered. Using the previously determined HL values for each record, we calculated the maximum HL for each of the selected polygons. The maximum value was selected in order to make sure the differences between patches would be evident, since selecting the mean HL could have made these variations less accentuated. The software Conefor 2.6 (Saura & Torné, 2009) was then used to determine the importance of each of the selected population patches. This software quantifies the importance of habitat areas and links, for the maintenance or improvement of landscape connectivity and identification and prioritization of critical areas (Saura & Torné, 2009). Conefor includes connectivity indices that have shown to outperform other existing indices and to be particularly suited for landscape conservation planning (Pascual-Hortal & Saura, 2006; Saura & Pascual-Hortal, 2007; Saura & Rubio, 2010; Saura *et al.*, 2011). These indices are based on spatial graphs and on the concept of measuring habitat availability and reachability, and include three separate fractions, intra, flux and connector (Saura & Torné, 2009). These indicate intrapatch connectivity, i.e. available habitat area provided by the patch

itself; flux (or number of connected patches) of the connections of a patch with all the other patches; and contribution of a patch to the overall connectivity between other patches, as connecting element or stepping stone, respectively (Saura & Rubio, 2010). This latter index is particularly relevant to identify patches that hamper populations fragmentation by promoting the connectivity of isolated patches.

Initially, through Linkage Mapper 1.1.0 (McRae & Kavanagh, 2011) we identified and mapped the linkages between our patches through least-cost paths. In order to do so, we used the previously defined distribution patches and the multivariate friction layer map, representing barriers to gene flow. Finally, the resulting maps of least-cost corridors between the patches and their mean HL values were input into Conefor. The software's output allowed us to identify the populations' intra, flux and connector patches, and, subsequently, the priority patches for *P. a. begognae*'s conservation.

RESULTS

Genetic diversity and population structure

The first STRUCTURE analysis identified two clusters (K=2) as the most likely number of genetic groups in our dataset (Fig. S3.1 and S3.2, Appendix S3, Supporting information III), corresponding to *P. a. begognae* and *P. a. auritus*. The threshold based on the effectiveness of our microsatellites to differentiate *P. a. begognae* and *P. a. auritus* was set to 0.885 and 0.791, respectively. Based on these values, our dataset was composed by 316 *P. a. begognae*. The geographical distribution of STRUCTURE individual assignments show that *P. a. auritus* were mainly restricted to a narrow range located in north-eastern Iberia, in the Pyrenees area. Results for the second STRUCTURE analysis (using 17 loci; see below) with *P. a. begognae* and admixed individuals identified only one population cluster (K=1 was the most likely number of genetic clusters; see Fig. S3.3, S3.4 and S3.5, Appendix S3, Supporting information III).

Two loci (Paus03 and Paus07) deviated from Hardy–Weinberg equilibrium at both *P. a. begognae* and *P. a. auritus* and were removed from analyses. The final dataset comprised 17 loci. All loci showed moderate to high polymorphism, and *P. a. begognae* and *P. a. auritus* exhibited high genetic diversity ($H_{E\text{-}begognae}$ = 0.771, $H_{E\text{-}auritus}$ = 0.816; see Table S3.1, Appendix S3, Supporting information III).

Species distribution modelling

After the removal of spatially correlated records, the final dataset considered for species distribution modelling comprised 144 records of *P. a. begognae*. Species distribution models, which had high predictive ability and did not overfit present data (training AUC = 0.9261±0.0026, test AUC = 0.8464±0.0694), showed that the species is mostly restricted to the mountainous and forested areas of Iberia, to the lower altitudes of the Pyrenees area and throughout northern Iberia with a patchy distribution in the central region mountains. The variables that contributed the most to the distribution models built were both climatic and landscape variables, demonstrating that *P. a. begognae*'s distribution seems to be restricted mainly by the proximity to forested areas, steeper slopes (>20º) and humid areas, occurring in temperate and rainy mountainous areas (Table 3.1). The resulting species distribution model and its plots of variable contributions and respective response curves can be found in Appendix S4, Supporting information III.

Table 3.1 - Set of final ecogeographical variables.

	Variable	**Code**
Climatic	Annual mean temperature (°C)	bio1
	Mean temperature of warmest quarter (°C)	bio10
	Precipitation of wettest quarter (mm)	bio16
	Precipitation of driest quarter (mm)	bio17
	Autumn mean temperature (°C)	tautumn
Topographical	Distance to slope > 20° (m)	dslope
Habitat	Distance to forests (m)	dforest
	Distance to water (m)	dwater
SDM	Species distribution model	sdm

The conversion of this species distribution model to polygons resulted in a total of 26 patches (with an average area of 2341 km^2±5254) to consider for connectivity analyses (Fig. S5.1, Appendix S5, Supporting information III).

Movement pattern analyses

Friction layers generated through Circuitscape for each of the 9 variables inputted (Table 3.1) can be found in Appendix S6, Supporting information III.

Landscape genetics

Of the calculated landscape resistance models, only the models for precipitation of wettest quarter, distance to forest, distance to slope and species distribution model had significant positive correlations with measures of genetic distance, in which the model with the highest correlation coefficient was distance to forest (R^2 = 0.004913948, $p < 0.0001$, Tables S7.1 and S7.2, Appendix S7, Supporting information III) The variables that remained significant in the final multivariate model (R^2 = 0.006218637) were distance to forest and distance to slope (Table 3.2).

Table 3.2 - Results of the final MRDM model.

	Estimate	**p value**
Final model	$5.861837E^{-17}$	0.0028
Distance to forest	-0.04960871	0.0001
Distance to slope	-0.0273405	0.0009

The multivariate friction layer built (Fig. 3.3) shows the potential movement pathways of *P. a. begognae* within northern Iberia, weighed by the variables that most affect its genetic differentiation, distance to forest and distance to slope. It is possible to observe that the main connectivity routes seem to be located along the northern and central mountains of Iberia.

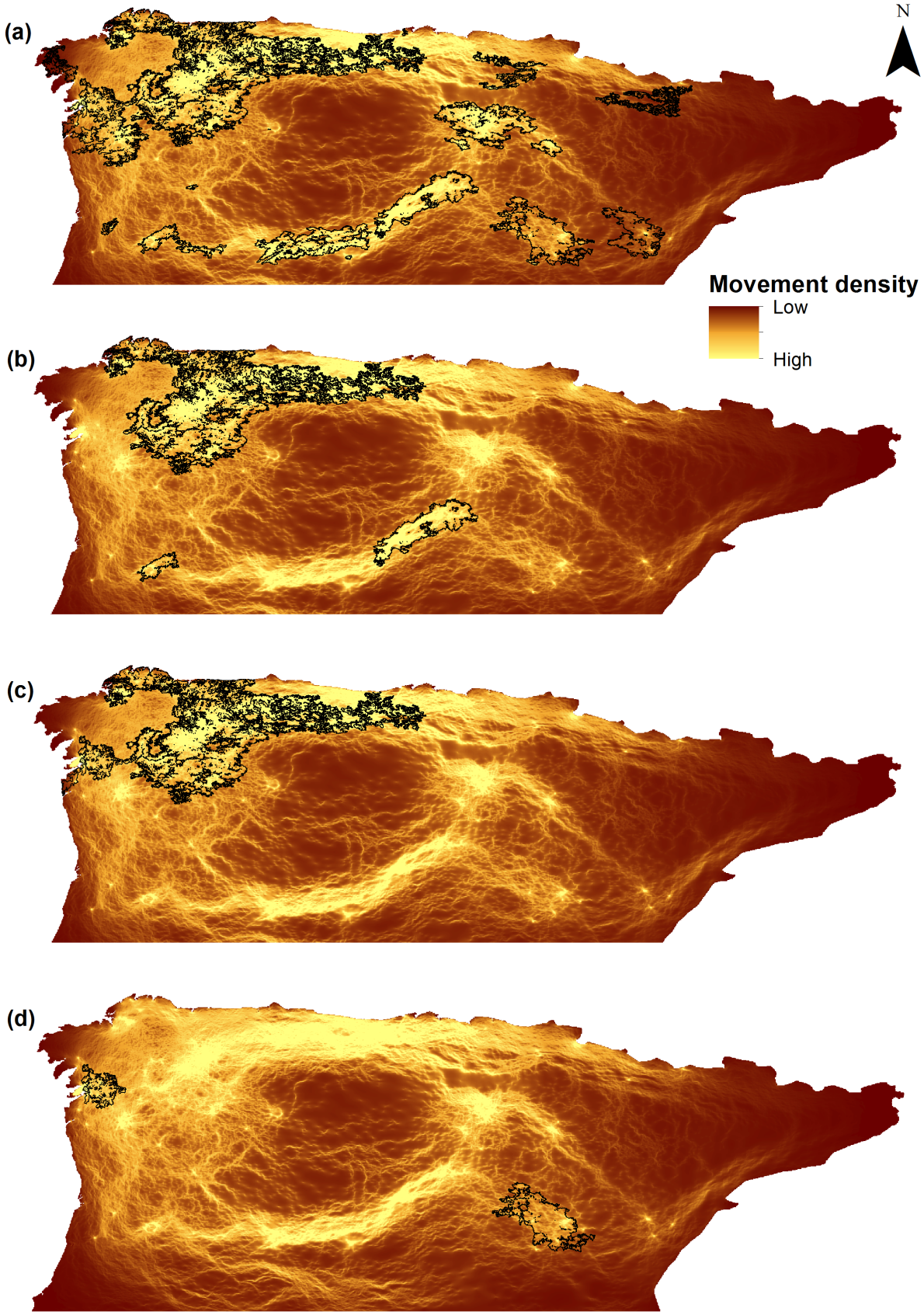

Figure 3.3 - Polygons representing each of the connectivity indices (a), intra (b), flux (c) and connector (d). In the background is the multivariate friction layer resulting of the final MRDM model.

Connectivity analyses

The most relevant patches for connectivity were identified (Table 3.3 and Fig. 3.3), in which node 11, the largest north eastern patch in Iberia, not only showed the highest importance in intrapatch connectivity, but also one of the highest percentages in dispersal flux. The other patches with high intrapatch connectivity were nodes 18 and 20 located in the patchy central region of Iberia. In eastern Iberia, node 8 presented the highest percentage in dispersal flux and was identified as the most important connector of all the patches, thus the most relevant to prevent population fragmentation. Node 10, also located in eastern Iberia, also presented as relevant for dispersal flux. Lastly, located in central western Iberia, node 26 showed the second highest importance as a connector patch.

Table 3.3 - Percentage contribution of the 26 nodes for each of the resulting CONEFOR indices. dPC (%) represents probability of connectivity.

Node	Intra (%)	Flux (%)	Connector (%)
1	0.257	0	0
2	3.732	3.283	0
3	4.769	0.001	0
4	1.912	3.283	0
5	4.464	7.399	0
6	2.341	0.077	0
7	0.691	0	0
8	4.493	**33.543**	**97.431**
9	5.985	0	0
10	5.959	**12.320**	0
11	**8.301**	**14.335**	0.158
12	5.825	0.299	0
13	2.598	0.299	0
14	6.048	0	0
15	4.988	6.828	0
16	0.106	0	0
17	0.981	0	0
18	**7.800**	0.033	0
19	1.542	0.996	0
20	**7.169**	2.629	0
21	4.150	2.629	0
22	3.158	0	0
23	3.863	0	0
24	5.906	0.033	0
25	0.281	5.582	0
26	2.678	6.427	**2.410**
dPC (%)	92.345	7.421	0.234

DISCUSSION

This study reveals the priority areas for the conservation of *P. a. begognae*, covering all its Iberian range. A multi-disciplinary framework was successfully implemented, integrating different methodologies for a common goal, to identify the potential at-risk populations of the species. Although *P. a. begognae*'s population does not seem to be fragmented, having no apparent connectivity issues between the population's patches, by applying this methodology we were able to detect which areas might be at risk of becoming isolated. If a single methodology landscape genetics approach were to be applied, we would not have been able to locate such areas, and therefore possible conservation threats would remain undetected. By integrating connectivity analyses, we were able to not only establish the most relevant patches for population maintenance, but also uncover the connector patches, which, if removed, would lead to the fragmentation and isolation of populations, leading to a higher susceptibility to local extinction.

We are aware of the limitations of the landscape genetics models calculated, since the resulting R^2 and estimate values are low. However, in our case, the subspecies considered does not seem to be genetically structured within the study area, therefore not fragmented. As such, it would be expected that the correlations between genetic structure and landscape variables are low. Furthermore, it is expected that if a colony-based approach would have been considered, the landscape genetics methodology would result in higher model fit, i.e. higher R^2 values. In fact, most population genetics literature supports the use of colony-based analyses or grouping individuals in populations (Paetkau *et al*., 1995; Pritchard *et al*., 2000; Corander *et al*., 2003). However, this approach is highly dependent on the sampling design and could not represent the species range in its totality, which was essential for the connectivity analyses. Only a few colony roosts of *P. a. begognae* are documented throughout the study area (Santos *et al.*, 2014), leaving most of the subspecies' distribution uncovered. An individual-based analysis allowed us to cover the entire subspecies' range, enclosing most of its biogeographical envelope and all environmental variability within the subspecies range. Correspondingly, Manel et al. (2007) showed that an individual-based approach is efficient in identifying spatial patterns of genetic diversity, and has the advantage of being able to be applied across a wide range of geographical scales.

The bat swarming sites phenomenon (Davis, 1964; Fenton, 1969; Thomas *et al.*, 1979), where individuals from different regions concentrate in a single area to mate, leads to lower genetic variability and higher relatedness in populations, as shown by (Veith *et al.*, 2004). This study

detected strong gene flow between *P. auritus* nursery colonies in Germany due to the swarming effect, demonstrating that sampling during the swarming months, late summer and early autumn, could mask genetic variability and any population structure that would otherwise be evident. However, our sampling occurred between April and October, supporting that the lack of genetic variability in P. a. begognae is actually due to its connectivity. Moreover, since the availability of forests showed to be of high significance for both *P. a. begognae*'s distribution and genetic variability, we can relate the high abundance of forested areas in the subspecies' distribution range to the lack of genetic structuring of its populations. The absence of major landscape fragmentation in the North of Iberia seems to be promoting gene flow for this subspecies.

General discussion

Genetic differentiation between different populations of *P. a. begognae* could not be detected in the study area, leading us to assume that this subspecies is genetically connected throughout its distribution range, with constant gene flow occurring between all population patches. Contrariwise, in previous studies, genetic differentiation between colonies of bats from the genus *Plecotus* was found in smaller geographical areas. In fact, Burland *et al*. (1999) and Veith *et al*. (2004) found significant differences in genetic structure between maternity colonies in Scotland and Germany, respectively. Moreover, Razgour *et al.* (2014) found high levels of genetic differentiation between colonies of the sister species *Plecotus austriacus* in the South of England, at the edge of range populations like the ones considered in our study. This recurrent phenomenon rests on the fact that *Plecotus auritus*, due to its wing morphology, presents very limited dispersal capabilities and small home range (Entwistle *et al.*, 1996). Nevertheless, Razgour *et al.* (2014) also uncovered that the colonies of *P. austriacus* present in the Iberian Peninsula were genetically less differentiated compared to the ones located in the South of England. These findings are more in line with our results and could be explained by the fact that, in Iberia, the occurrence of suitable habitat is more prevalent. In fact, in the same study, landscape genetics analyses determined that habitat suitability, mainly the presence of grasslands, was the main driver of genetic differentiation of *P. austriacus*. Concordantly, meadows and grasslands are widespread through the whole Iberian Peninsula, suggesting possible habitat continuity. In our study, *P. a. begognae*'s genetic structure showed to be significantly affected by the distance to suitable habitat, mainly forests and high slope areas, and these landscape characteristics are predominant throughout the North of the Iberian Peninsula.

P. a. begognae's population does not seem to be fragmented, although it seems that the most predominant connectivity routes are located in the northern and central mountainous regions of Iberia, where the broadest population of the subspecies is located. Even though all the population patches are connected through routes throughout the study area, the subspecies seems to avoid connecting through the locations with less suitable habitat (higher distances to forests and slopes), as there is less density of routes in the centre (North Submeseta) and most eastern (Ebro River Basin and South of the Pyrenees) regions of northern Iberia. Nevertheless, the methodology used allowed us to identify the population patches that are at most risk of isolation, located in the most western and eastern regions of the subspecies' distribution range. Even though the patches located in the western regions (West Galicia) are closer to the largest source population, it seems that there are several smaller population patches in the coastal areas that could be at risk of isolation. At higher risk are the most eastern (South Iberian Range) located patches, where there is lower density of connecting routes and are further from the source populations. The identified connector patch in this region, if removed or even diminished, could lead to the isolation of these populations, increasing the risk of local extinction.

Several connectivity methodologies are being widely used in conservation planning to prevent or reduce population fragmentation in other taxa (Mateo-Sánchez *et al.*, 2014; Ziółkowska *et al.*, 2014; McGuire *et al.*, 2016).

Circuit theory (McRae & Beier, 2007; McRae *et al.*, 2008) is often applied (Cianfrani *et al.*, 2013; Koen *et al.*, 2014) and frequently coupled with landscape genetics to infer on both connectivity and gene flow (Garroway *et al.*, 2011; Polato *et al.*, 2011; Van Strien *et al.*, 2012; Epps *et al.*, 2013; Dupas *et al.*, 2014; Marrotte *et al.*, 2014; Razgour *et al.*, 2014). Nevertheless, patch and link connectivity analyses, such as Conefor, is rarely combined with the previously mentioned methodologies since it is mainly used for habitat management and fragmentation studies.

Implications for conservation

The multi-disciplinary framework presented allows a comprehensive spatial analysis of the population structure and connectivity and has the potential of applicability for other taxa. In a first approach, it is able to establish the amount of populations that comprise the data, then it determines the possible distribution range and the considered ecological variables that are shaping it. Afterwards, it defines the main routes and barriers to gene flow, and which are its

limiting variables. Finally, it delimits the relevant population patches, showing which areas are connected or prone to isolation, and which patches should be prioritized for conservation.
By identifying the significant population patches, source and connectors, and uncovering possible at-risk populations, this methodology allows for the application of pre-emptive conservation measures. Consequently, we propose the implementation of conservation planning actions to promote the connectivity of P. a. begognae's populations within the north and centre of the Iberian Peninsula. Essentially, forest management and preservation in Galician coastal areas and in the south-western Iberian Range, should be the focus of conservation programmes in order to prevent fragmentation and isolation of the at-risk populations located in these areas. Since P. a. begognae is a newly-discovered subspecies, with an unknown conservation status, efforts should be made to protect and maintain its populations. Our results reinforce the need to protect the forested habitats in the Iberian Peninsula for effective conservation of this bat.

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SUPPORTING INFORMATION III

Appendix S1 – Genetic methodology

Microsatellite genotyping

Microsatellite loci were amplified in three multiplex reactions according to Table S1.1. Total reaction volume was 10 ul, including 5 ul of the QIAGEN PCR Master Mix, 1 ul of primer mix, and 2 ul of DNA. Forward primers were M13-tailed to follow a fluorescent labelling protocol (Blacket *et al*., 2002). A negative control was always included in all reaction sets to monitor possible contaminants. PCR Thermo-cycling conditions are described in Figure S1.1.

Table S1.1 – Microsatellite loci genotyped in our study. Three multiplex panels were used using four dyes. Allele ranges and references for microsatellite development are given.

Loci name	Multiplex	Dye	Allele range	Reference
Paus20	1	FAM	105-131	Razgour *et al*. 2013
Paus15	1	NED	129-159	Razgour *et al*. 2013
Paur06	1	PET	190-248	Burland *et al*. 1998
Paus17	1	FAM	199-223	Razgour *et al*. 2013
Paus19	1	NED	226-271	Razgour *et al*. 2013
Paus04	1	VIC	285-389	Razgour *et al*. 2013
Paus06	2	VIC	190-248	Razgour *et al*. 2013
Paus18	2	FAM	160-192	Razgour *et al*. 2013
Paus14	2	NED	134-192	Razgour *et al*. 2013
Paus08	2	PET	165-209	Razgour *et al*. 2013
Paur05	2	FAM	261-297	Burland *et al*. 1998
Paus09	2	NED	279-287	Razgour *et al*. 2013
Paur01	3	NED	157-185	Burland *et al*. 1998
Paus12	3	FAM	151-189	Razgour *et al*. 2013
Paus01	3	VIC	213-281	Razgour *et al*. 2013
Paus02	3	NED	193-233	Razgour *et al*. 2013
Paus05	3	FAM	244-268	Razgour *et al*. 2013

Steps	Temperature	Time	Cycles
1	95°C	15 min	
2	95°C 58°C down 0.5°C per cycle 72°C	3 sec 1 min 45 sec	13
3	95°C 52°C 72°C	30 sec 1 min 45 sec	27
4	60°C	30 min	

Figure S1.1 – Thermocycling conditions used to amplify loci included in multiplexes 1, 2 and 3 described in Table S1.1.

Appendix S2 – Ecogeographical variables

Table S2.1 – Set of 15 variables used in the species distribution model.

Type	Variable	Code
Climatic	Annual Mean Temperature	bio1
	Mean Diurnal Range	bio2
	Mean Temperature of Warmest Quarter	bio10
	Mean Temperature of Coldest Quarter	bio11
	Precipitation of Wettest Quarter	bio16
	Precipitation of Driest Quarter	bio17
Topogra--phical	Altitude	dem
	Maximum slope	slopemax
	Distance to slope >20°	disl20
	Distance to maximum slope >20°	dislmax20
Habitat	Land cover:	
	Agriculture 1	
	Orchards 2	
	Forested agriculture 3	
	Forest 4	
	Coniferous 5	land
	Shrubs 6	
	Bare 7	
	Urban 8	
	Water 9	
	Eucalyptus forests 10	
	Distance to forests	diforest
	Distance to agriculture	diagric
	Distance to water bodies	diwater
	Distance to eucalyptus forests	dieucal

Appendix S3 – Population genetic structure

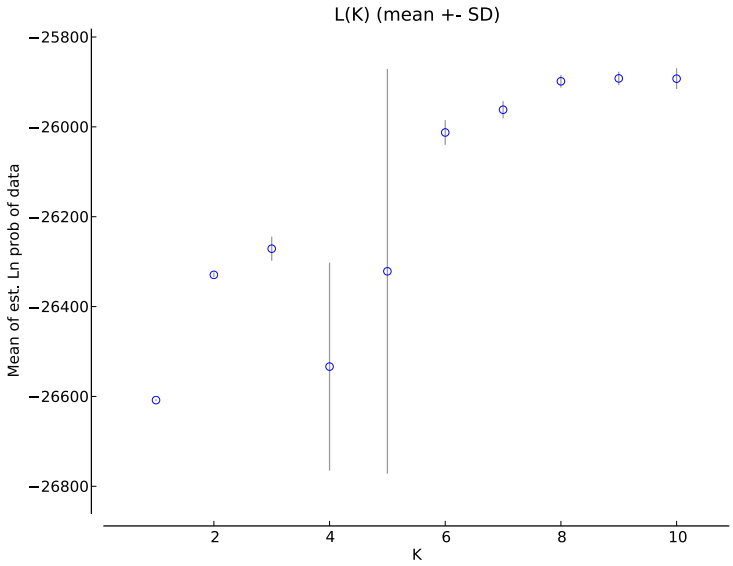


Figure S3.1 – Plot of mean likelihood L(K) and variance per K value from STRUCTURE on a dataset containing 373 individuals (*Plecotus auritus auritus* and *Plecotus auritus begognae*) genotyped for 17 polymorphic microsatellites. Plot was obtained through STRUCTURE HARVESTER (Earl & Von Holdt, 2012).

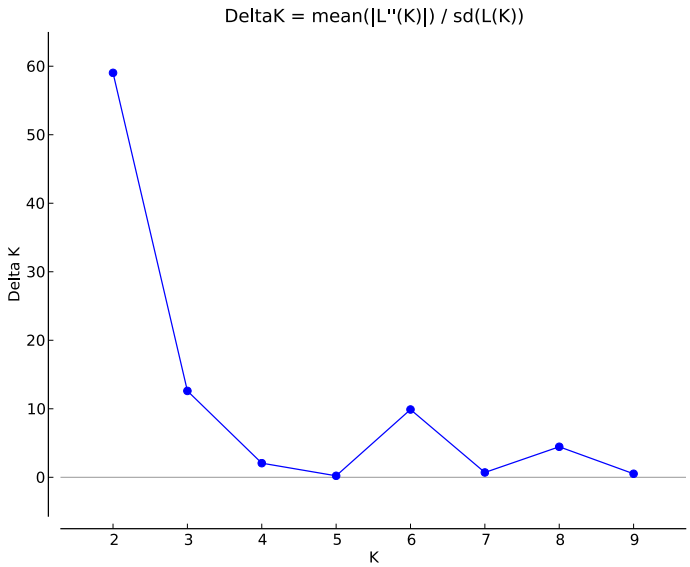


Figure S3.2 – Evanno *et al*. (2005) plot for detecting the number of K groups that best fit the data (data as in Fig. S3.1). Plot was obtained through STRUCTURE HARVESTER (Earl & Von Holdt, 2012).

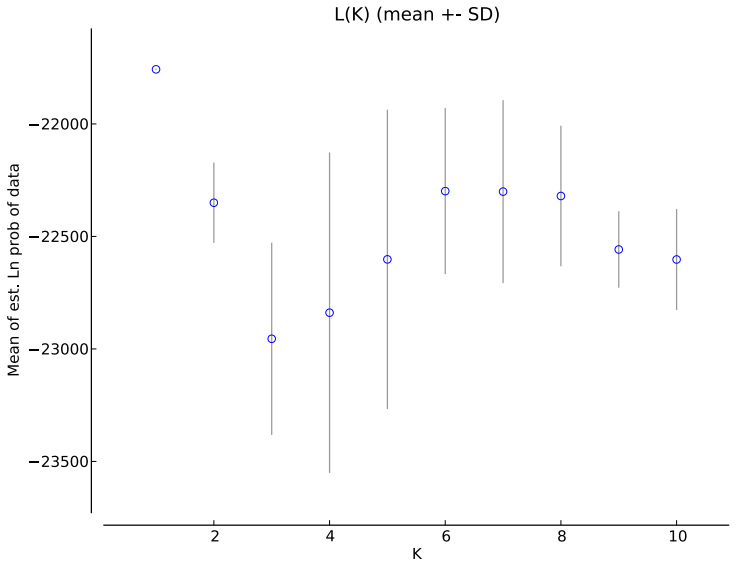


Figure S3.3 – Plot of mean likelihood L(K) and variance per K value from STRUCTURE on a dataset containing 316 individuals (*Plecotus auritus begognae*) genotyped for 17 polymorphic microsatellites. Plot was obtained through STRUCTURE HARVESTER (Earl & Von Holdt, 2012).

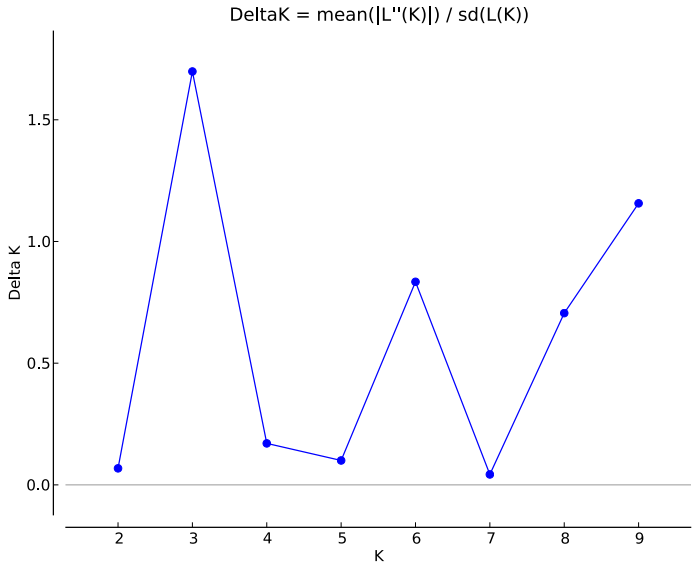


Figure S3.4 – Evanno *et al*. (2005) plot for detecting the number of K groups that best fit the data (data as in Fig. S3.3). Plot was obtained through STRUCTURE HARVESTER (Earl & Von Holdt, 2012).

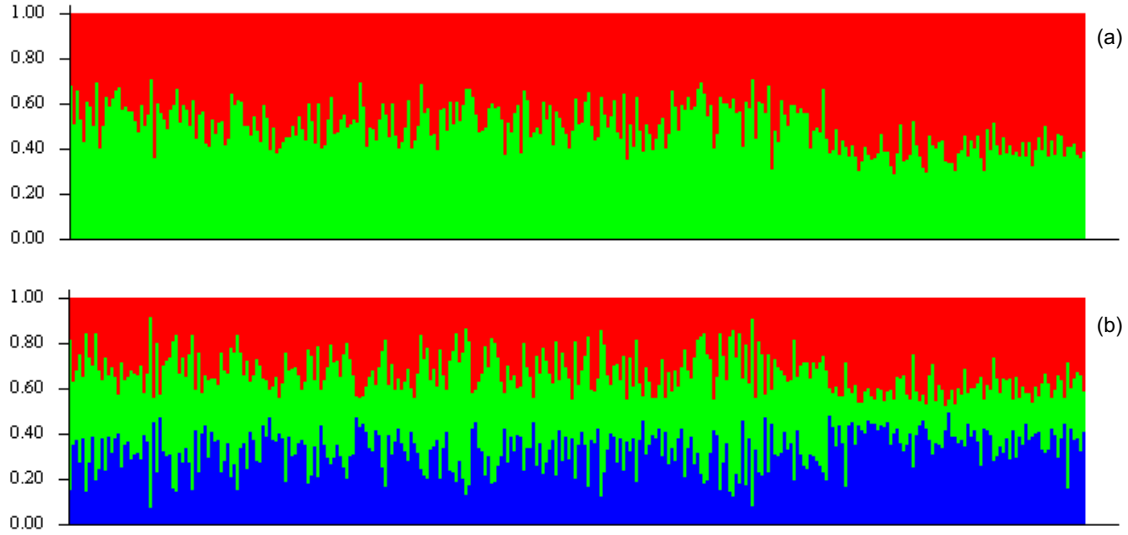


Fig. S3.5 – Examples of STRUCTURE bar plots illustrating the lack of population structure of *P. a. begognae* (data as in Fig. S3.3) for K=2 (a) and K=3 (b).

Table S3.1 – Number of alleles per locus (NA), observed heterozygosity (HO) and expected heterozygosity (HE) for each locus, for *Plecotus auritus begognae* and *Plecotus auritus auritus*.

	P. a. begognae			***P. a. auritus***		
Loci	**Na**	**H_O**	**H_E**	**Na**	**H_O**	**H_E**
Paus01	12	0.838	0.816	11	0.686	0.790
Paur01	8	0.628	0.796	11	0.784	0.820
Paus02	9	0.741	0.777	13	0.902	0.880
Paus04	16	0.889	0.871	19	0.765	0.910
Paus05	15	0.963	0.898	26	0.800	0.943
Paur05	7	0.630	0.650	13	0.863	0.869
Paus06	10	0.728	0.811	13	0.882	0.883
Paur06	16	0.444	0.904	24	0.922	0.931
Paus08	11	0.790	0.805	20	0.804	0.919
Paus09	3	0.432	0.475	4	0.520	0.541
Paus12	6	0.716	0.742	9	0.843	0.800
Paus14	4	0.474	0.574	7	0.529	0.663
Paus15	11	0.889	0.852	15	0.941	0.880
Paus17	14	0.716	0.800	14	0.745	0.835
Paus18	12	0.790	0.846	14	0.863	0.889
Paus19	17	0.863	0.922	22	0.765	0.922
Paus20	5	0.481	0.573	11	0.353	0.394

Appendix S4 – Species distribution modelling

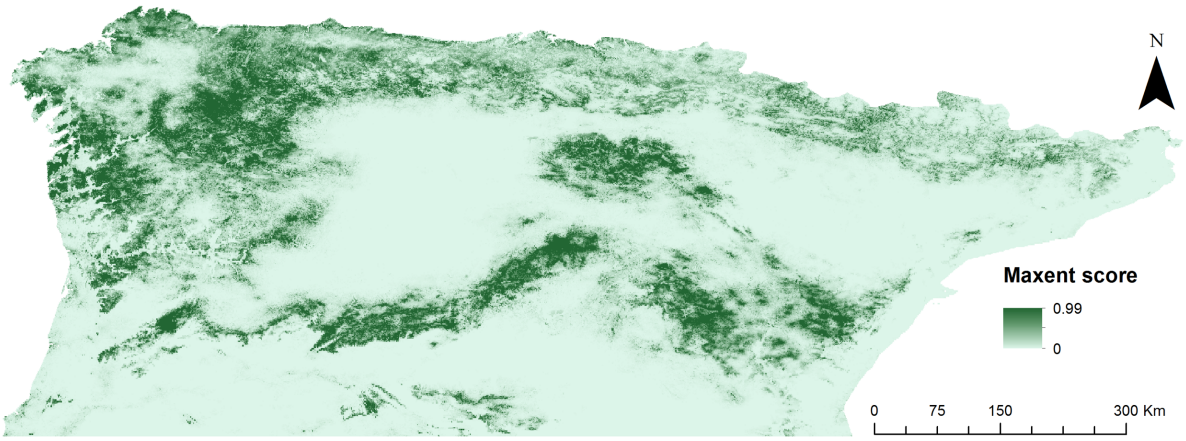


Fig. S4.1 – Map of the potential distribution of *Plecotus auritus begognae*. Maxent score indicates 0 for no suitability and 0.99 for high suitability.

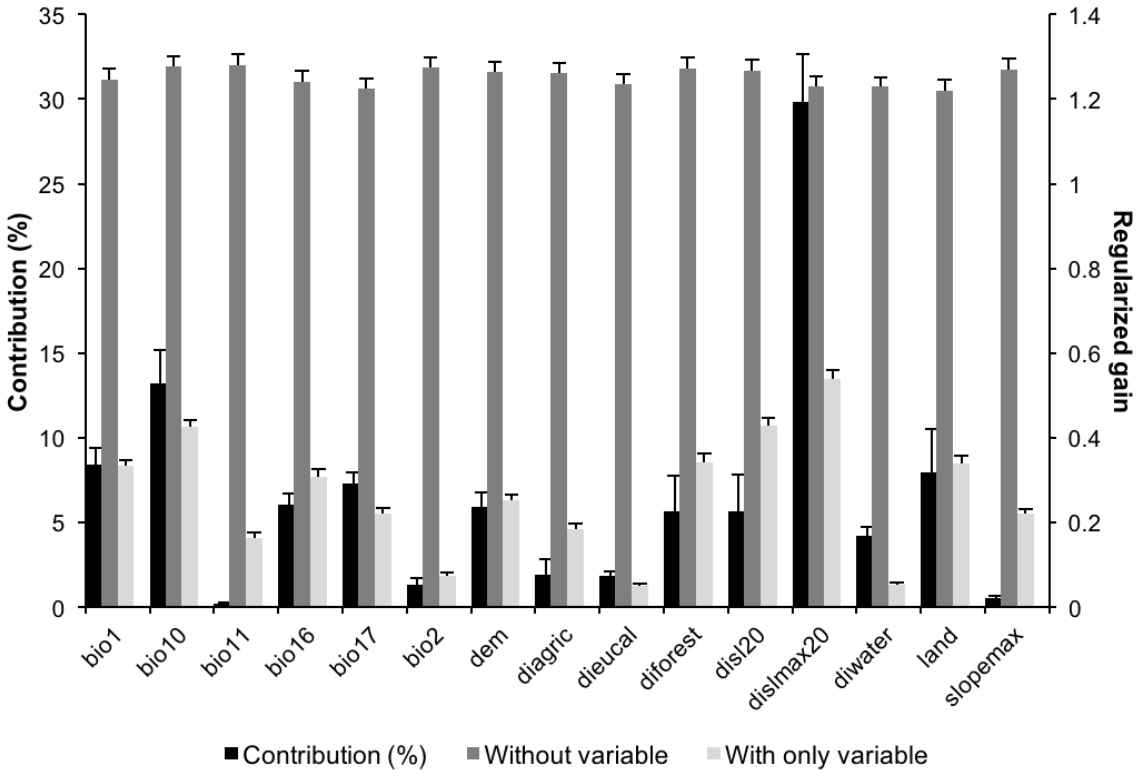


Fig. S4.2 – Graph representing variable importance for the *Plecotus auritus begognae* distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S2.1.

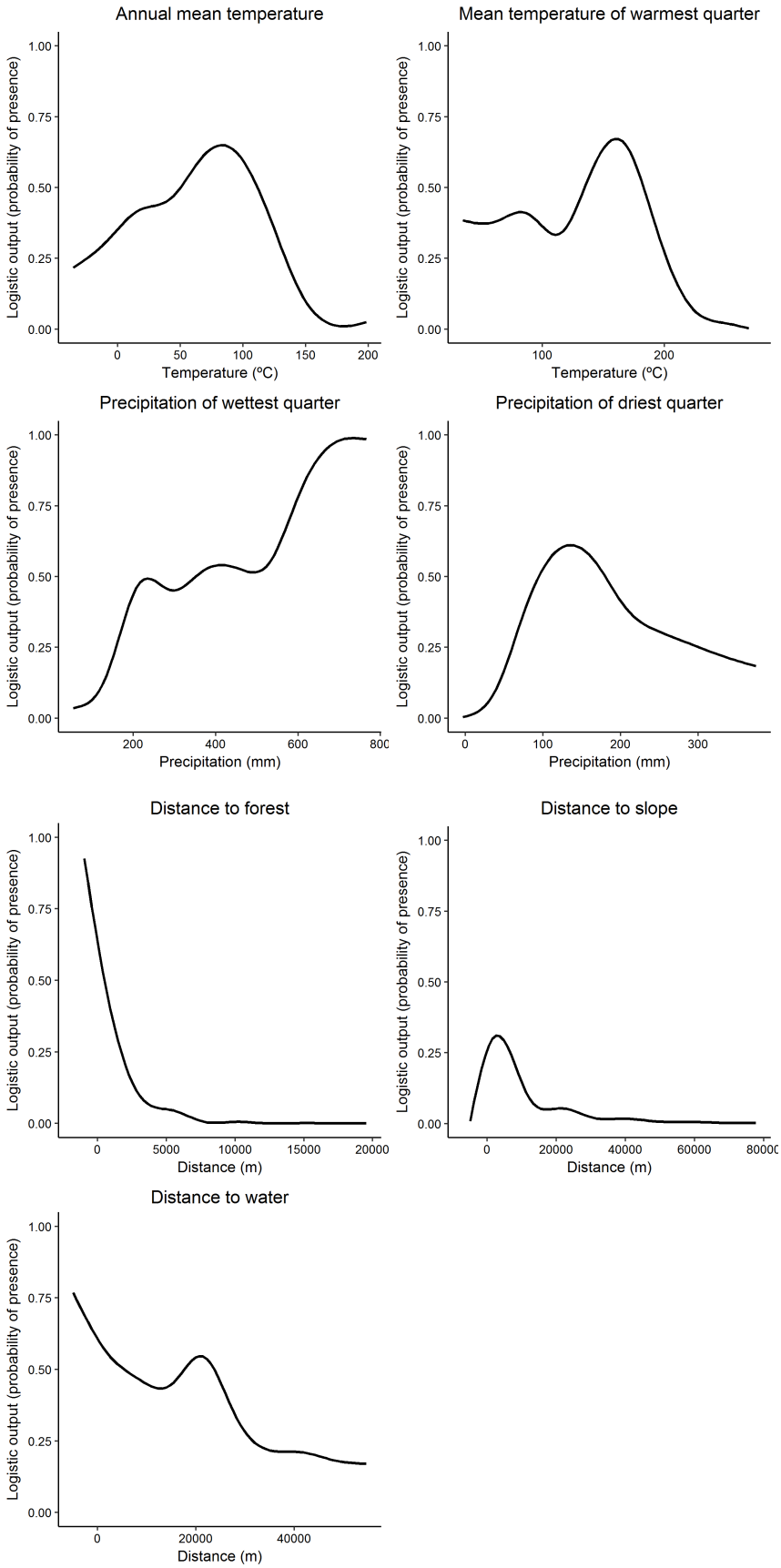


Fig. S4.3 – Response curves of the environmental variables most related to the distribution of *Plecotus auritus begognae*.

Appendix S5 – Distribution patches

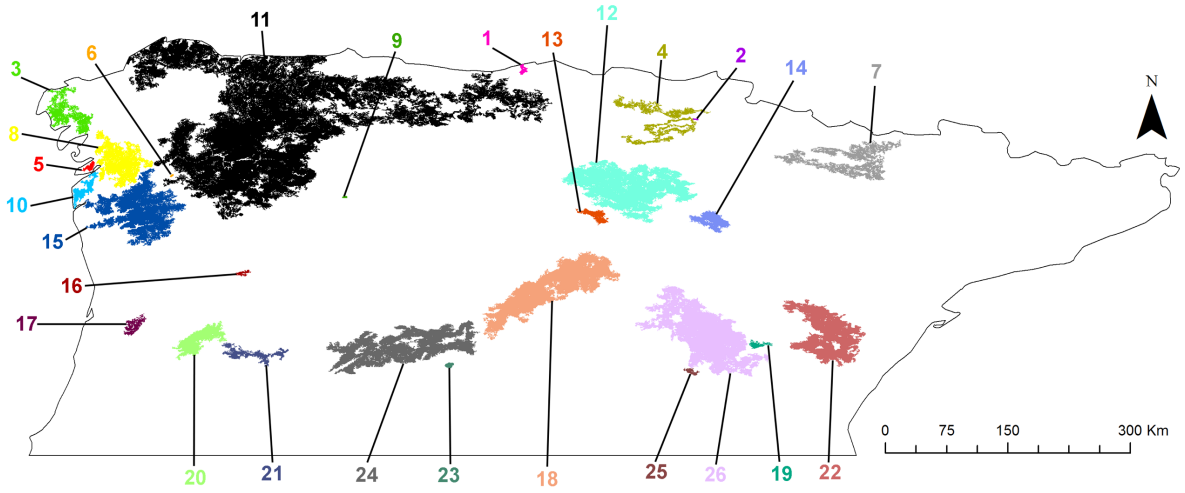
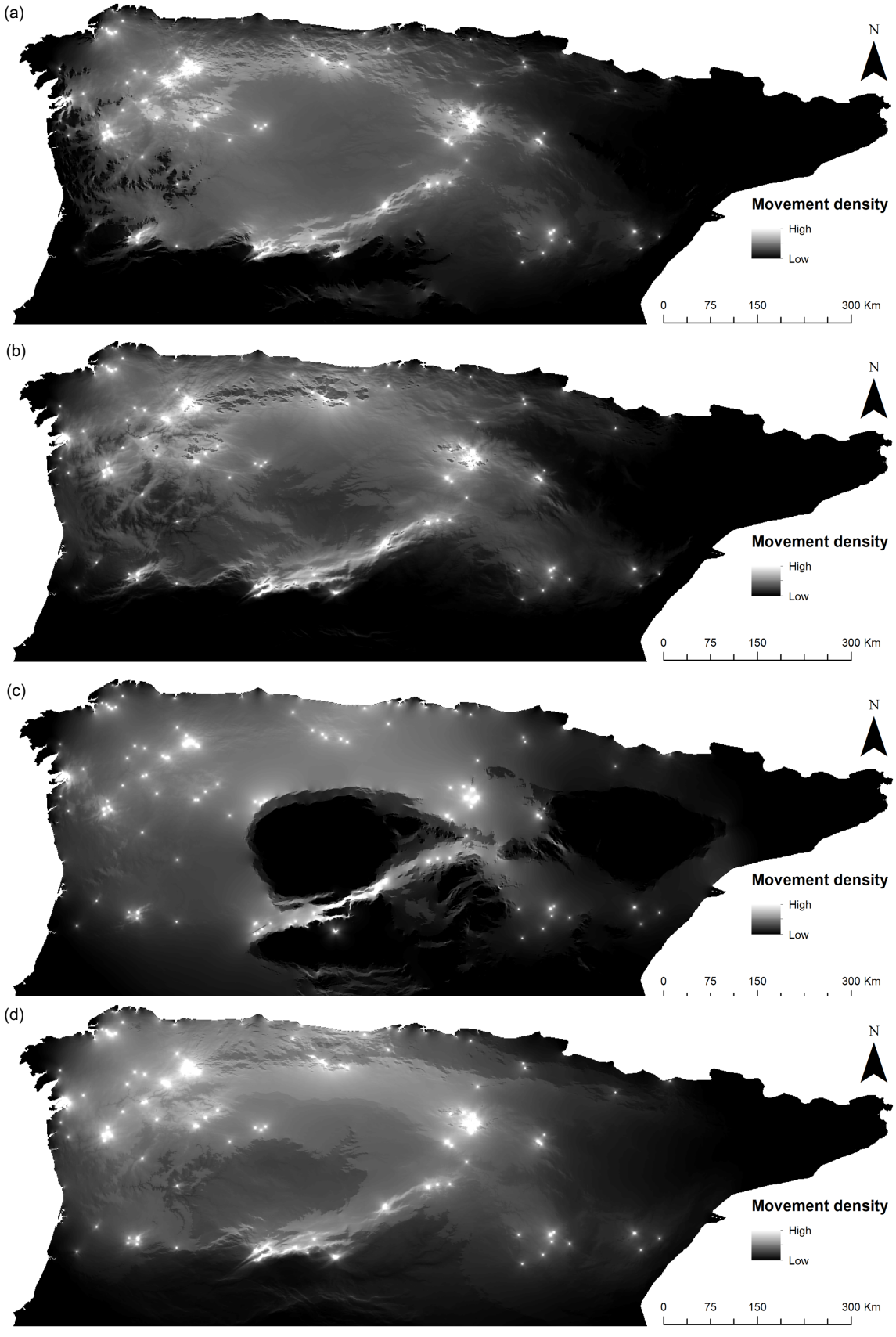
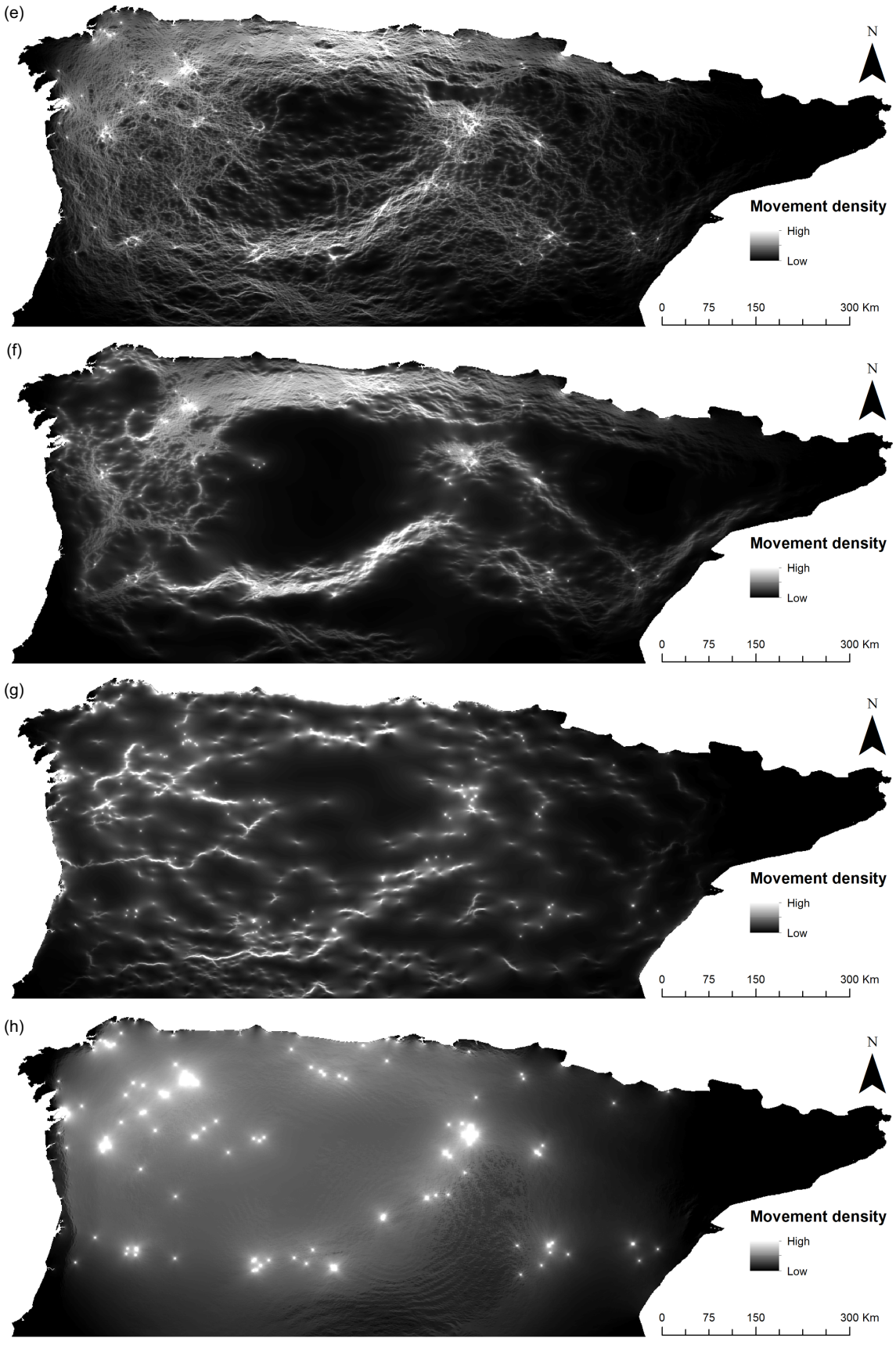


Fig. S5.1 – Map of the 26 distribution polygons (patches) for *Plecotus auritus begognae*.

Appendix S6 – Movement pattern analyses




(e)
N
Movement density
High
Low
0 75 150 300 Km
(f)
N
Movement density
High
Low
0 75 150 300 Km
(g)
N
Movement density
High
Low
0 75 150 300 Km
(h)
N
Movement density
High
Low
0 75 150 300 Km

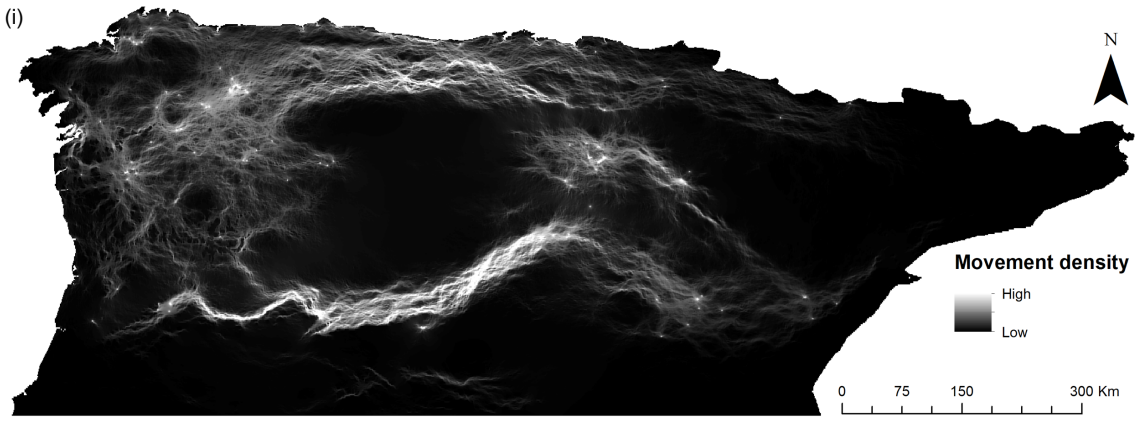


Fig. S6.1 – Potential movement pathways of *Plecotus auritus begognae* within the study area. Cumulative current maps generated by Circuitscape to indicate movement density between records for (a) annual mean temperature, (b) mean temperature of warmest quarter, (c) precipitation of wettest quarter, (d) precipitation of driest quarter, (e) distance to forests, (f) distance to slope > 20°, (g) distance to water, (h) autumn mean temperature and (i) species distribution model.

Appendix S7 – Landscape genetics

Table S7.1 – Univariate multiple regressions on distance matrices (MRDM) models. Variable abbreviations are available in Table S2.1. Statistical significance: *p < 0.05.

Model	Estimate	P-value	R^2	F
bio1	6.89E-03	0.440	4.13E-05	2.058
bio10	-2.47E-03	0.613	1.85E-05	0.920
bio16	-8.94E-03	0.048*	3.48E-04	17.301
bio17	9.45E-06	0.710	9.45E-06	0.470
dforest	-5.89E-02	0.0001*	4.91E-03	245.765
dslope	-3.99E-02	0.0001*	3.04E-03	151.543
sdm	-3.85E-02	0.0001*	2.46E-03	122.803
tautumn	-5.83E-03	0.578	1.89E-05	0.939
dwater	-9.29E-03	0.169	1.57E-04	7.806

Table S7.2 – Multivariate multiple regression on distance matrices (MRDM) model. Variable abbreviations are available in Table S2.1. Statistical significance: *p < 0.05.

Model	Estimate	P-value	R^2	F
Int	5.98625E-17	0.0023	0.00625731	78.33895
bio16	0.001755187	0.7684		
dforest	-0.05171083	0.0001*		
dslope	-0.03023789	0.0154*		
sdm	0.00452411	0.7577		

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CHAPTER 5

IMPACTS OF CLIMATE CHANGE ON *PLECOTUS AURITUS BEGOGNAE*

PAPER IV - USING LANDSCAPE GENETICS AND CLIMATIC MODELLING TO MINIMIZE THE IMPACT OF CLIMATE CHANGE ON THE POPULATIONS OF *PLECOTUS AURITUS BEGOGNAE* IN THE IBERIAN PENINSULA

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ABSTRACT

Understanding how biodiversity will respond to future climate change is a major conservation and socio-economic challenge. Climate change is predicted to force several species to shift their ranges in pursuit of suitable conditions. Research on this topic has been mainly focused in the use of species distribution modelling that identifies the most suitable future areas for a species but does not consider species' connectivity and gene flow patterns which are essential for population viability. Understanding species responses to climate change requires an interdisciplinary perspective, combining ecological, molecular and environmental approaches. This study aims to use a multi-disciplinary approach methodology, combining climatic modelling with landscape genetics, as a predictive tool to assess how species will shift their ranges and inform conservation measures that will facilitate movement when species are faced with climate change. We demonstrate how this framework can be applied to species with limited dispersal abilities, by using the bat *Plecotus auritus begognae* as a model species. This framework was applied to climate change scenarios for 2050 and 2070. Our main results indicated that the climate variable precipitation of the wettest quarter is the main driver affecting *P. a. begognae*'s genetic connectivity and that the most important range shift pathways are located along the eastern and northern coasts of the Iberian Peninsula. Most of the species' distributional range will suffer a severe contraction, with several suitable areas disappearing entirely. By identifying the potential pathways for future range shifts of the species, we can determine the priority areas for the application of conservation management measures to increase landscape connectivity and facilitate movement.

INTRODUCTION

The issue of climate change has been increasingly on the spotlight during the 21st century, from political decision-making to scientific research, and the consensus seems to be that it's becoming one of the main threats to biodiversity worldwide (IPCC, 2013). Several species have already been affected by climate change, having presented changes in phenology, geographical range, ecological relationships and even local survival rates (Pounds *et al.*, 1999; Parmesan & Yohe, 2003; Root *et al.*, 2003; Sanz *et al.*, 2003; McMahon & Burton, 2005; Peñuelas *et al.*, 2013). Extreme events that advent from climate change, such as extended periods of droughts, thermal variations or increasing sea-level values, present new challenges for species capability to respond to these pressures (Hoffmann & Sgrò, 2011), and therefore, increasing their vulnerability.

Recent research has been focusing on the potential impacts of climate change on global biodiversity, predicting that one of the most prominent effects may be extreme range shifts, forcing the movement of a great number of animal and plant species from their original areas of occurrence to new unoccupied regions (McLachlan *et al.*, 2005; Araújo & Rahbek, 2006; Araújo *et al.*, 2006; Kirilenko & Sedjo, 2007; Huntley *et al.*, 2008; IPCC, 2014). Associated to this process are negative effects on populations, such as local extinctions and fragmentation, which could lead to lower genetic variability and high levels of inbreeding in populations (Thomas *et al.*, 2004; Ezard *et al.*, 2006) thus compromising the local viability of populations.

One of the most popular fields to address these impacts are spatially explicit methods, such as ecological niche models or species distribution models (ENMs/SDMs). These tools have shown to be quite effective in determining species habitat requirements and potential distribution ranges under future climate changes (Guisan & Thuiller, 2005). As such, they are widely applied in predicting how climate change will affect future species distribution patterns (Thomas *et al.*, 2004), identifying species more susceptible to future changes (Pacifici *et al.*, 2015) and predicting extinction risks (Urban, 2015). However popular, these methods include several assumptions that affect their outcomes, such as niche conservatism (Rebelo *et al.*, 2012), where species are expected to maintain their ecological and habitat preferences in the new predicted areas of occupation. Additionally, most climate change studies do not seem to integrate genetic data to support their predictions (Gotelli & Stanton-Geddes, 2015), or take into account evolutionary processes (Thuiller *et al.*, 2013). Most of these analyses do not take into account possible species adaptation capability, assuming species will most likely shift their distribution ranges rather than tolerate or even adapt to new conditions (Buckley *et al.*, 2010), and assume that the projected new areas of occurrence are always reachable, not taking into account possible movement barriers (Araújo *et al.*, 2006). Landscape connectivity will affect the ability of species to shift their distribution ranges and disperse to new predicted suitable areas (Razgour, 2015) and, most of climate change studies either assume no dispersal or unlimited dispersal scenarios (Bateman *et al.*, 2013), failing to take into account how landscape connectivity species movement patterns.

Landscape genetics is one of the latest methodologies in combining landscape ecology and population genetics (Holderegger & Wagner, 2008), relating the effects of landscape structure on the movement of organisms to patterns of genetic variation. This framework allows for the identification of species' movement patterns according to their genetic variability, representing gene flow barriers and dispersal corridors (Manel *et al.*, 2003; Storfer *et al.*, 2007).

In this study, we combined landscape genetics with climatic modelling, in order to predict future suitable areas and potential colonization routes. By applying a multi discipline approach, we are able to better understand not only what will affect future species distribution patterns, but also what bioclimatic variables are most related to species genetic variability. This allows us to more accurately predict species' movements in future climate change scenarios, by determining the most probable gene flow routes. The Iberian subspecies of the European Brown long-eared bat, *Plecotus auritus begognae*, was used as a case study in this work. It is known to occur in the northern part of the Iberian Peninsula (Santos *et al.*, 2014; see also Chapter 4) and it is characterised by its limited dispersal capabilities (Burland *et al.*, 1999). This particular trait makes it an ideal subject to apply the methodology proposed, as it is unlikely that this species will disperse further from its current distribution range, allowing us to focus the analyses in the Iberian Peninsula. Moreover, since it is circumscribed to Iberia, we are able to model the subspecies' entire distribution range, making sure we cover the whole scope of ecological and landscape requirements. Moreover, it has been suggested that future climate change will strongly affect bats occurring in southern Europe, probably causing major range shifts (Rebelo *et al.*, 2010; Razgour *et al.*, 2013). The Iberian Peninsula in particular has been the case study in several climate change studies (Gordo & Sanz, 2006; Benito Garzón *et al.*, 2008; Razgour, 2015). This advents from the fact that a major Pleistocene glacial refuge for European biodiversity was located in Iberia and that it is where climate change impacts are predicted to be most severe (Milly *et al.*, 2005; Dai, 2011).

In our research, we aimed at understanding what will be *P. a. begognae*'s distribution and dispersal routes in future climate change scenarios and what bioclimatic characteristics will shape those patterns. Mainly, we aimed to answer the following questions:

1. How will predicted climate change affect *P. a. begognae*'s distribution?
2. What climatic variables shape current genetic population structure of P. a. begognae?
3. Where will the most suitable routes for *P. a. begognae*'s dispersal be located and connectivity status between suitable habitats?
4. Which areas currently occupied by *P. a. begognae* are going to be most affected?

MATERIALS AND METHODS

Study area and sampling design

The study area was the northern Iberian Peninsula, previously described as the location of the main distribution range of the focal subspecies, *P. a. begognae* (Fig. 4.1) (Santos *et al.*, 2014; see also Chapter 4). Samples were collected through mist netting and roost trapping sessions for over a course of 10 years, in which, for each specimen, a non-lethal tissue sample from the wing membrane was biopsied in the field and later stored in ethanol for laboratory analyses. All samples were subject to molecular analyses to determine species identification and respective taxonomical placement, through the genotyping of 17 highly polymorphic microsatellites. The genetic methodology used in this study is outlined in the Methods section of Chapter 4 and in Appedix S1 and S3, Supporting information III. This genetic and geographic data was then used to compute genetic and geographic distance matrices between *P. a. begognae*'s records, respectively, to later use for landscape genetics analyses. Genetic distance was calculated through GenAlEx 6.5 (Peakall & Smouse, 2012) and geographic distance through the packages "maps" (Becker & Wilks, 2017) and "reshape2" (Wickham, 2007) in R version 3.3.2 (R Core Team, 2012).

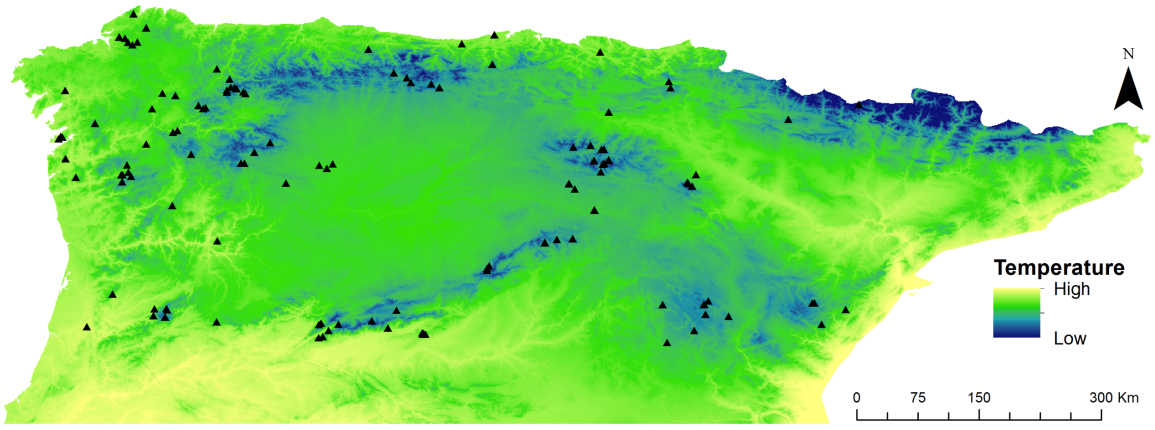

Figure 4.1 - Mean annual temperature of the study area and *Plecotus auritus begognae*'s records (black triangles). Each symbol may correspond to more than one location.

Bioclimatic variables and future scenarios

An initial set of 19 bioclimatic variables were obtained through Worldclim (http://www.worldclim.org) and cropped for our study area (Table S1.1, Appendix S1, Supporting information IV). In a first approach, in order to decrease the number of variables for the species distribution models, the highly correlated ones were eliminated by first calculating a correlation matrix and then selecting the pairs with correlations above 0.80 (Dormann *et al.*, 2013). Also with the purpose of fine-tuning the species distribution models, spatial autocorrelation between the records of *P. a. begognae* was eliminated through the spatially rarefy occurrence data tool in SDMtoolbox (Brown, 2014). All variables had a resolution of approximately 1km (30 arc-seconds) and respective calculations were made in ArcGIS 10.1 (ESRI, 2012).

Global Climate Models/General Circulation Models (GCMs) projections and respective representative concentration pathways (RCPs) from CMIP5 (IPPC Fifth Assessment) were also obtained through Worldclim. Initially, 13 GCMs were selected based on the availability of data for RCP8.5 for the decades of 2050 and 2070. The decision to select the highest RCP value was supported on the fact that we aimed for the modelling procedures to reflect the scenarios most concordant with current climate change predictions. Afterwards, in order to objectively select the most influential GCMs for our study area, we used the k-means clustering approach described by (Casajus *et al.*, 2016).

Species distribution modelling and resistance surfaces

Using the determined set of uncorrelated bioclimatic variables and the set of spatially independent records of *P. a. begognae*, species distribution models (SDMs) were calculated using the maximum entropy presence-only modelling approach presented by Maxent 3.3.3k (Phillips *et al.*, 2006). This approach has proven robust in previous studies with this and similar species (Rebelo *et al.*, 2010; Razgour *et al.*, 2014; Santos *et al.*, 2014; see also Chapter 4), due to its reliability when dealing with presence-only, scarce and limited datasets (Hernandez *et al.*, 2006; Wisz *et al.*, 2008; Elith *et al.*, 2010). SDMs were ran in auto features, with 20 cross-validation model replicates and logistic output format. This procedure allowed us to determine the bioclimatic variables that most contributed to the current distribution of *P. a. begognae*, and therefore, the variables that would most significantly shape the subspecies' distribution in future climate change scenarios. The resulting most relevant variable selection was then used to calculate multivariate SDMs, projecting for each of the most significant

GCMs, for 2050 and 2070. This was accomplished by using *P. a. begognae*'s presence records and the previously determined most relevant bioclimatic variables, projecting them for each of the GCMs. Projections for each GCM for both decades were then used to build two ensemble (Araújo & New, 2006) projections, one for each decade, joining all of the GCM projections of 2050 and then all of the GCM projections of 2070. This procedure allowed us to have a better understanding of the predicted future distribution of *P. a. begognae* in both decades. We are aware that only the ecological conditions of *P. a. begognae* are considered for the models' calculations instead of considering the whole range of conditions occupied by the species *Plecotus auritus*. This could be considered partial-niche modelling, which is unsuitable to be used for climate change projections because the partial models fail to predict the whole range of conditions a species may be able to thrive under predicted climate change (Elith *et al.*, 2010). However, (Alberdi *et al.*, 2014) showed that spatial models produced better predictions when considering the separate modelling of each population or lineage. In our case, we are considering the whole subspecies for analysis, hence following the best procedure for model projections.

With the purpose of obtaining continuous resistance surfaces of each relevant bioclimatic variable, univariate SDMs were calculated. These univariate models were ran in auto features, with cumulative output format and no replicates, by using the records of *P. a. begognae* in turn with each of the most relevant bioclimatic variables. In order to also obtain resistance surfaces for the future climate change scenarios, this method was then repeated for the same bioclimatic variables, but instead, projecting for each of their future counterparts in each GCM, for 2050 and 2070. Ensemble resistance surfaces for each of the bioclimatic variables were then built, by joining all of the GCM projections for the same variable, in each decade. This procedure allowed us to obtain composite resistance surfaces of each of the most relevant bioclimatic variables, for the decades of 2050 and 2070. Each resistance surface was then scaled from 1 to 101, where lower values, i.e. lower probability of species' occurrence, were assigned to movement barriers and higher values, i.e. higher probability of species' occurrence, were assigned to features which promote gene flow.

Movement pattern analyses

In order to determine the subspecies' possible movement patterns, i.e. gene flow routes, in present and future climate change scenarios, friction layers were built using the movement pattern analyses software Circuitscape 4.0.5 (McRae, 2006; McRae *et al.*, 2008). Circuitscape predicts patterns of movement and gene flow among individuals or populations, based on

electronic circuit algorithms, which have proven to outperform other approaches, such least-cost paths, as it considers effects of all possible pathways across a landscape simultaneously (McRae & Beier, 2007). Previously calculated resistance surfaces and respective datasets were input in Circuitscape and then ran using the pairwise modelling mode.

In order to obtain more reliable results when calculating the friction layers for the future climate change scenarios, three sets of different movement pattern analyses were built for the subsequent landscape genetics analyses, by using the subspecies' records and the previously calculated multivariate SDMs (Razgour, 2015). The first, hereafter "route present", to characterise which were the gene flow routes occurring in the present subspecies' distribution. For this, the dataset of all present *P. a. begognae*'s records was used. The second analysis, hereafter "route 2050", aimed at determining which were the gene flow routes between the populations located in present suitable areas and the populations located in suitable areas in 2050. In this analysis, we used the records located in suitable areas in the present, but unsuitable in 2050, as the source population and the records located in suitable areas in the present and in 2050, as the destination population. Finally, in the third analysis, hereafter "route 2070", we intended to define the gene flow routes between the populations in suitable areas in 2050 and the populations in suitable areas in 2070. As such, we used the records located in suitable areas in 2050, but unsuitable in 2070, as the source population and the records located in suitable areas in 2050 and 2070, as the destination population. In order to ensure all possible location records were incorporated in the analyses, a buffer of 5km (known species home range; Entwistle *et al.*, 1996) was built around the ensemble SDM's predicted suitable areas, before building the datasets.

For the route present, univariate SDMs of each of the most relevant bioclimatic variables were input as conductance surfaces, and the complete dataset of *P. a. begognae*'s distribution records was input as the node location file. For the routes 2050 and 2070, ensemble univariate SDM projections for each of the most relevant bioclimatic variables were input as conductance surfaces, and the previously built datasets of source and destination populations were input as the node location files. This approach allowed us to obtain friction layers of each of the most significant bioclimatic variables for the present, route 2050 and route 2070.

Landscape genetics

With the purpose of determining which bioclimatic variables were shaping *P. a. begognae*'s movement patterns, i.e. gene flow, landscape genetics analyses were performed using

multiple regressions on distance matrices (MRDMs) (Legendre *et al.*, 1994). This landscape genetics methodology has previously shown to perform well with this type of datasets (Razgour *et al.*, 2014; see also Chapter 4) and is widely used in landscape genetics analyses (Garroway *et al.*, 2011; Epps *et al.*, 2013; Marrotte *et al.*, 2014; Roffler *et al.*, 2016). For these analyses, the resistance matrices previously produced in Circuitscape for each of the most relevant bioclimatic variables, were imported in R along with the genetic and geographic distance matrices. All matrices were standardized from 0 to 1, using the "scales" package (Wickham, 2016). Since genetic structure is known to be affected by geographic distance (Dyer *et al.*, 2010; Razgour *et al.*, 2014), geographic variation was removed from all of the resistance and genetic distance matrices. By removing this effect, we make sure that the models calculated reflect the role that the bioclimatic resistance elements are having in structuring the subspecies' patterns of gene flow. This was accomplished through the calculation of linear models of the geographic distance against each of the bioclimatic resistance variables and genetic distance, to determine its residuals (Dyer *et al.*, 2010). Subsequently, MRDMs were calculated using the package "ecodist" (Goslee & Urban, 2007). In a first approach, to determine which of the bioclimatic variables had significant correlations with genetic divergence, univariate MRDMs of the genetic distance for each of the variables were calculated. Then, we proceeded to calculate multivariate MRDMs of the genetic distance, using the significant ($p<0.05$) variables previously obtained in the univariate models. This approach allowed us to determine the final bioclimatic drivers that were influencing genetic divergence and gene flow of *P. a. begognae*.

In a final approach, previously built multivariate ensemble SDMs for the present, 2050 and 2070 were used to determine how the predicted suitable areas of *P. a. begognae* will change in the future climate change scenarios. To do so, we first reclassified the SDMs built using the maximum training sensitivity plus specificity logistic threshold value (Liu *et al.*, 2013) obtained in Maxent, and then overlapped the predicted suitable areas for each time period.

RESULTS

Species Distribution Models

An initial set of 316 records of *P. a. begognae* was considered. After eliminating duplicate records and spatial autocorrelation analyses, 144 records of the subspecies remained. The first set of SDMs built with the present dataset and nine uncorrelated bioclimatic variables (Table 4.1), allowed us to determine the most relevant variables affecting current *P. a. begognae*'s predicted distribution, and, therefore, which bioclimatic variables would be most relevant when facing climate change scenarios. These were annual mean temperature (bio1), mean temperature of the warmest quarter (bio10) and precipitation of the wettest quarter (bio16) (for variable contributions see Fig. S2.1, Appendix S2, Supporting information IV).

Table 4.1 - Set of nine bioclimatic variables used in the species distribution model.

Variable	**Code**
Annual Mean Temperature	bio1
Mean Diurnal Range	bio2
Isothermality	bio3
Temperature Seasonality	bio4
Mean Temperature of Warmest Quarter	bio10
Mean Temperature of Coldest Quarter	bio11
Precipitation Seasonality	bio15
Precipitation of Wettest Quarter	bio16
Precipitation of Driest Quarter	bio17

By using the k-means clustering approach (Casajus *et al.*, 2016), the most relevant GCMs in our study area were determined. These were HadGEM2-ES (HE), GISS-E2-R (GS), MPI-ESM-LR (MP) and INMCM4 (IN).

The multivariate SDMs were built using the three previously determined most relevant bioclimatic variables (bio1, bio10 and bio16), and then projected for each of the four most significant GCMs (HE, GS, MP and IN) for both the decades of 2050 and 2070. These SMDS had high predictive ability and did not overfit present data (mean training AUC = 0.8794±0.0034). The resulting four projections for each decade, were used to build the ensemble models and, therefore, allowed us to have a more precise conceptualization of species predicted distribution in both decades. The shifting of the subspecies' distribution

patterns can be observed both in the decades of 2050 and 2070, mainly by contraction of its predicted distribution area (Fig. 4.2).

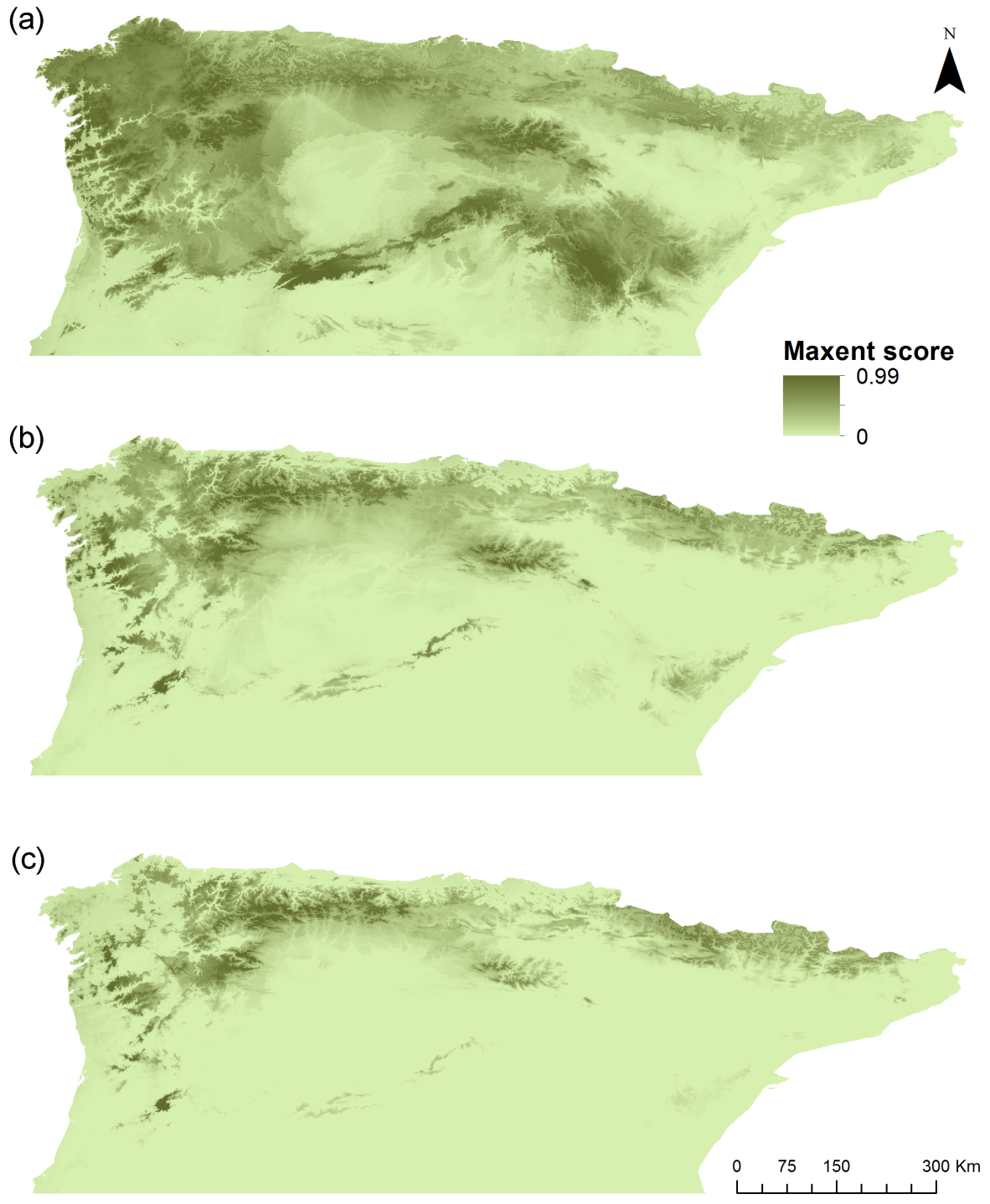

Figure 4.2 - Map of the potential distribution of *Plecotus auritus begognae* for the present (a), ensemble distribution model for 2050 (b) and ensemble distribution model for 2070 (c). Maxent score indicates 0 for no suitability and 0.99 for high suitability.

Landscape genetics

The landscape genetics models calculated allowed us to determine which bioclimatic variables, as resistance surfaces, were shaping *P. a. begognae*'s genetic divergence throughout the study area. The only variable that showed was significant (R^2 = 0.001664252, p = 0.0003) was precipitation of the wettest quarter. Circuitscape analyses of this variable showed us the subspecies' main possible routes of dispersal, i.e. gene flow. In the present, the main routes seem to be located in centre Iberia (Central Iberian Range) and in the northern regions (Cantabrian Mountains) (Fig. 4.3a). In 2050 and 2070, most routes concentrate in the western and northern coastal areas of Iberia (Fig. 4.3b, 4.3c).

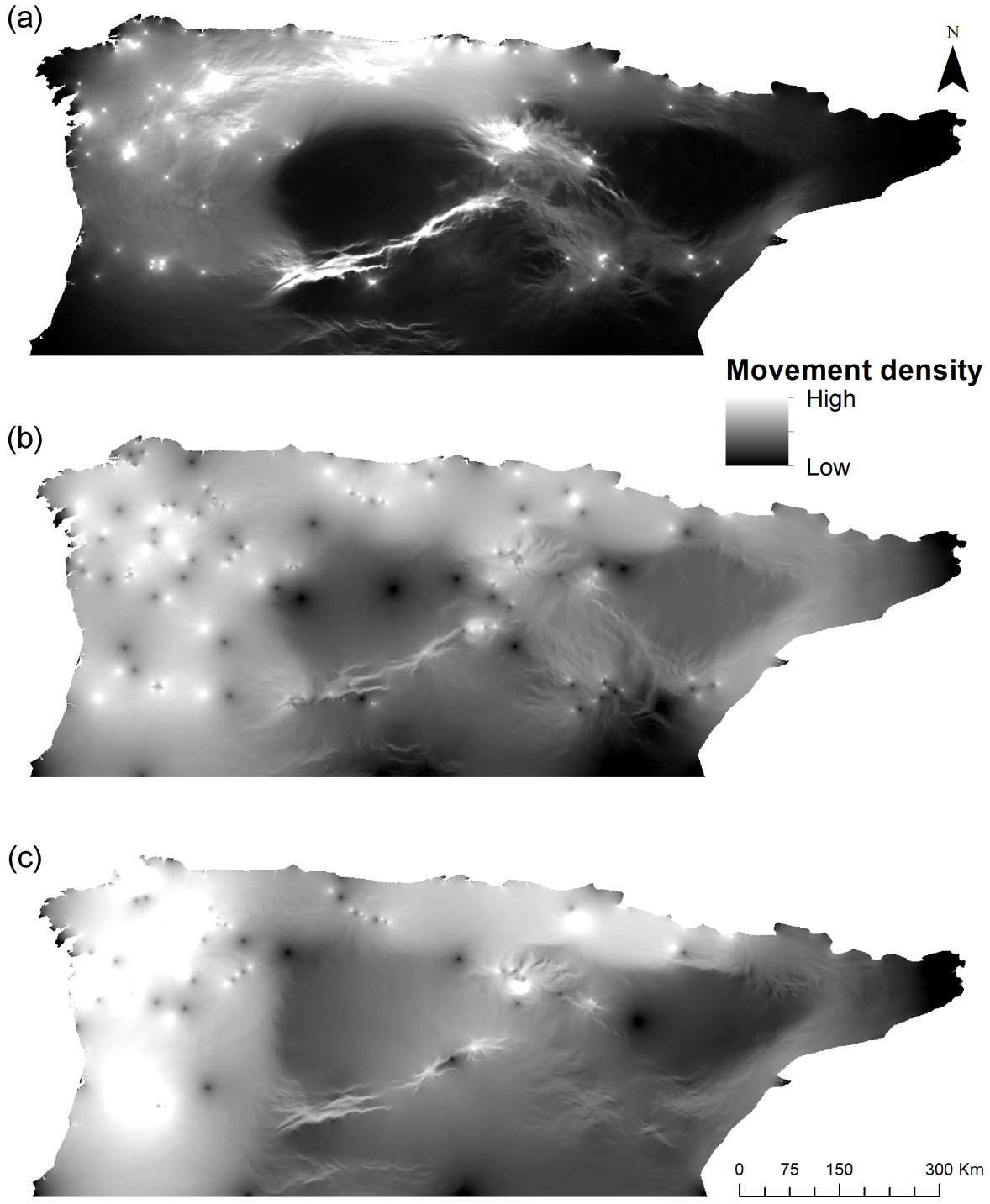

Figure 4.3 -Potential movement pathways of *Plecotus auritus begognae* within the study area. Cumulative current maps generated by Circuitscape to indicate movement density between records the route present (a), route 2050 (b) and route 2070 (c), using precipitation of the wettest quarter as the resistance surface.

Suitable areas

By overlapping the ensemble SDMs, we are able to observe the shifting patterns of *P. a. begognae*'s predicted distribution areas (Fig. 4.4). The most prominent change observed is the massive contraction of the subspecies' predicted distribution range. The centre and centre-eastern (Central Iberian Range and Iberian Range) distribution patches seem to be absent in future predictions, and most of the north-western distribution area shows an evident contraction. A small region in the Central Iberian Range seems to be preserved in the 2050 predictions, but vanishes in the decade of 2070. Some distribution areas in the north (Cantabrian Mountains) and northwest (Pyrenees), seem to suffer a slight northwards shift. The areas that seem to remain stable throughout the projections are some distribution patches in the northwest and north of Iberia (Galicia and West Cantabrian Mountains), and small patches in north-western coastal areas (Galicia), centre-west region (Serra da Estrela) and centre-north (north Iberian Range).

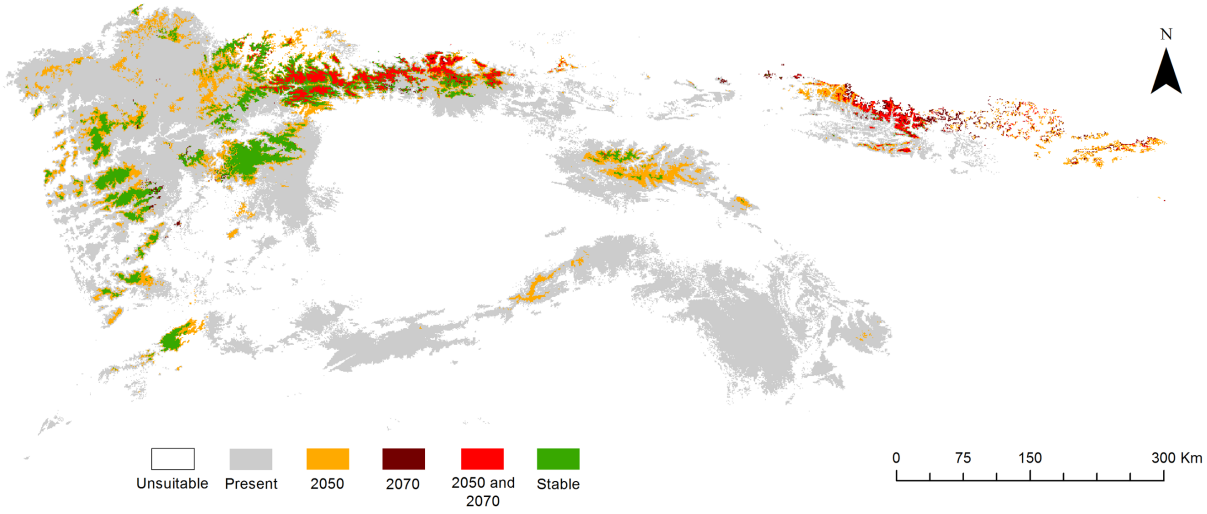

Figure 4.4 - Overlapped predicted distribution patches for *Plecotus auritus begognae*. Grey areas are suitable in the present, orange areas are suitable in projections for 2050, dark red areas are suitable in projections for 2070, bright red areas are suitable in projections for 2050 and 2070 and green areas are suitable in the present, 2050 and 2070.

DISCUSSION

In this study, we manage to predict not only which will be the suitable areas for the occupation of the subspecies *P. a. begognae* in future climate change scenarios, but also their possible colonization routes. We used a multi-disciplinary approach, by combining climatic modelling with landscape genetics, to determine the subspecies' suitable distribution areas in the future and the bioclimatic variables that most relate to the its genetic divergence and therefore, its gene flow.

Our results indicate that *P. a. begognae* may suffer serious threats with predicted climate change scenarios, as the majority of the predicted suitable areas for the subspecies appear to suffer a significant contraction, decreasing substantially in size and most of the suitable patches will ultimately disappear. However, we managed to identify and highlight the stable suitable areas for the subspecies, and which will be the most likely connection routes taken. In some of the areas we observe a slight range shift north, such as in the Pyrenees, but in most of the distribution range we witness a severe contraction of the areas, as such, most of the routes seem to be located facing inwards from the previously broader distribution areas. Most of the patches that show to remain stable throughout the decades of climate change scenarios, are located in the mountainous regions of north-western and north centre Iberia. As such, it seems that the north-western regions, Galicia and the Cantabrian Mountain range, and the mountain areas of central Portugal and the north of the Iberian Range, are the most important habitat patches when it comes to species preservation through time. Of special concern are the areas in central and central eastern Iberia, where climate change could eliminate entirely suitable climatic conditions for *P. a. begognae*.

Impact of climate change on *P. a. begognae*

The bioclimatic variables that seemed to be shaping *P. a. begognae*'s distribution, demonstrated that this subspecies seems to be restricted mainly in areas with temperate climate with high pluviosity. Moreover, our results show that precipitation of the wettest quarter was the most significant bioclimatic variable shaping *P. a. begognae*'s genetic variability in Iberia. The north of Iberia is biogeographically mainly Eurosiberian (Sillero *et al.*, 2009; Romo & García-Barros, 2010), being characterised with lower temperatures and high pluviosity when compared with the southern Mediterranean regions. *P. a. begognae*'s distribution is restricted to a more Eurosiberian climate (Santos *et al.*, 2014), as such, it would be expected that climatic conditions characteristic of this biogeographical regions would be shaping its gene flow.

SDMs have been widely used to project species distributions to climate change scenarios (Thomas *et al.*, 2004; Guisan & Thuiller, 2005). However, several issues with climate modelling have been pointed out in other studies, such as the inability to integrate ecological and evolutionary processes, like biotic interactions and evolutionary adaptions (Thuiller *et al.*, 2013). These processes could ultimately reshape species predicted distribution patterns, in particular adaptations, which might enable species to survive in areas with climatic conditions outside their known ecological niche. Moreover, basing species distribution patterns on bioclimatic variables alone could be considered problematic, since it is known that other ecogeographical variables may affect species distributions (Heikkinen *et al.*, 2006), and also that there are uncertainties associated with GCM projections. In fact, when comparing our bioclimatic distribution models to the ones in Santos *et al.* (2014) and those in Chapter 4, we can observe a slight over-prediction of the species distribution range. However, due to their usual broader suitable area predictions, climatic envelopes have been demonstrated to reflect the 'best case' scenario, particularly when projecting to future climate change (Rebelo & Jones, 2010). Therefore, from the conservation point of view and considering that our models predicted a severe contraction of the predicted future bat range, these over-predictions have no impact on the projected connectivity routes, hence on its proposed conservation measures. Essentially, the true possible scenarios may actually be significantly worse than our predictions, since it could be a lot more restricted.

Bioclimatic models have been successfully used to predict species potential distribution ranges under climate change (Hijmans & Graham, 2006) and seem to provide a reliable approximation to the possible effects of climate change on species distributions (Pearson & Dawson, 2003; Guisan & Thuiller, 2005). The use of Maxent also aids in providing more robust results, as it considers pseudo-absences in its calculations, implying that predictions are more likely to reflect the realized niche of the species considered (Zaniewski *et al.*, 2002). Moreover, the use of continuous variables in the landscape genetics analyses prevents biases from parameterisation of resistance surfaces (Koen *et al.*, 2012; Razgour, 2015; see also Chapter 4).

Bats and climate change

Bats may be less susceptible than other mammals to the negative effects of range shifting due to climate change, since they possess volant capabilities and can more easily relocate to new locations (Scheel *et al.*, 1996). Nevertheless, particularly for tree-dwelling bats such as *P. a. begognae*, climate change poses a special threat by limiting the availability of suitable

roosts and specific foraging habitats, those currently being the most limiting resources for bats (Rodrigues *et al.*, 2003; Russo & Jones, 2003; Russo *et al.*, 2004). It was previously determined (Santos *et al.*, 2014; see also Chapter 4) that *P. a. begognae*'s distribution is highly dependent on forests. The rapid contraction of suitable distribution will restrict these bats to currently existing forests mainly in northern Iberia. In the case of the northward expansions to new suitable areas, the bat may face serious challenges in their colonization due to the existence of scarce forested patches in higher altitudes. The development of mature forests takes decades to centuries to fully mature (McLachlan *et al.*, 2005), hence it is unlikely that suitable forests for *P. a. begognae* may develop until the end of the 21st century.

Both temperature and habitat changes may affect species' phelonogy, this being one of the main concerning effects of climate change. This is of special concern, since many bat species' phenology is highly related with temperature, such as breeding, gestation, hibernation and metabolic rate (Racey *et al.*, 1987; Webb *et al.*, 1995; Sanz *et al.*, 2003; Thomas *et al.*, 2004) and the increase of extreme weather events like droughts may pose additional challenges for the bats' survival (Amorim *et al.*, 2015).

There are several studies that show that climate change may present a severe threat for bats. (Rebelo *et al.*, 2010; Razgour *et al*., 2013) determined that most of the European bat species in temperate and boreal regions will have little to no suitable habitat in the future. It has also shown that the southern range species, such as in the Mediterranean south of Iberia, could have their predicted distribution ranges increased, having more availability of suitable areas. This, however, could present a severe threat to the more northern species, such as *P. a. begognae*, which will have much more restricted distribution ranges, and could suffer from changes in ecological interactions that could change fundamental ecosystem processes in unpredictable ways (Walther *et al.*, 2002). (Hughes *et al.*, 2012) also demonstrated the most of the Southeast Asian bat species will lose their entire niche space with climate change, leading to their probable extinction.

Moreover, it would seem that climate change has already had several effects on European bats, such as northwards range shifts (Sachanowicz *et al.*, 2006) and changes in gestation periods (Ibáñez, 1997). These changes may become more evident or even worsen, as changes in the climate manifest, particularly with higher predicted temperature increases (Jones & Rebelo, 2013).

Advantages of landscape genetics in climate change studies

Using landscape genetics as a predictive tool combined with SDMs allows for the identification of potential gene flow routes, whose loss could affect the ability of species to shift their range into future suitable areas in response to climate change. These gene flow routes can be interpreted as a proxy for potential dispersal pathways for the species and aid in identifying which areas may become isolated. Population fragmentation and isolation may lead to a reduction of gene flow between subpopulations, leading to genetic bottlenecks, elevated levels of inbreeding, population divergence and reduced genetic diversity (Templeton *et al.*, 1990; Fahrig, 2003). Several studies on a diversity of taxa have shown that the lack of connectivity is of major concern to species' maintenance and survivability (Jump & Peñuelas, 2006; Tournant *et al.*, 2013; Mateo-Sánchez *et al.*, 2014).

By using the presented approach, we are able to inform on conservation measures in order to include the proposed gene flow routes, therefore promoting connectivity between future isolated populations. The traditional approach of predicting species' future suitable areas of occurrence, and applying conservation measures solely to those locations, could not be enough to maintain population connectivity and could undermine its future viability.

Implications for conservation

Our analyses show a considerable contraction of the distribution range for *P. a. begognae* in Iberia. Most of the distribution patches located in the centre region of the Iberian Peninsula will ultimately disappear by 2070 projections, and the north and north-western distribution patches will suffer a major area reduction. For conservation management purposes, an important strategy is to identify focal areas for increasing landscape connectivity to facilitate species movements (Heller & Zavaleta, 2009). Using this approach, we were to identify which will be the stable suitable areas and which are the subspecies' main dispersal pathways. As such, we propose that conservation efforts should be focused on the mountainous regions of the centre-west and north-western Iberia. Since this subspecies' distribution range seems to be mainly restricted to forested mountainous areas (see Chapter 4), the preservation of mature forests to minimize the impacts of climate change in these populations is of high importance. This way we are able to guarantee that, in future scenarios, there will be suitable habitats and roosts for the destination population areas. Of particular concern are the distribution patches of centre and centre-east Iberia, which seem to either disappear or become completely isolated. Connections between these at-risk populations and the more

stable areas should be increased. This can be achieved by ameliorating habitat and roosting conditions in these routes, by the placement of forested patches and/or artificial roosting sites, such as bat boxes (Entwistle *et al.*, 1997). This way, we would be able to improve the connecting routes between the populations from the unsuitable areas in the future to the projected suitable areas, making them easier to reach.

With this approach, we are able to build pre-emptive conservation measures to mitigate the impacts of climate change in these bat populations. Moreover, the methodology used shows strong applicability for other taxa and geographical regions, being able to be applied in other contexts to develop management actions for future climate-change impacts.

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SUPPORTING INFORMATION IV

Appendix S1 – Bioclimatic variables

Table S1.1 – Set of 19 bioclimatic variables used in the initial species distribution model.

Variable	Code
Annual mean temperature	bio1
Mean diurnal range	bio2
Isothermality	bio3
Temperature seasonality	bio4
Maximum temperature of the warmest month	bio5
Minimum temperature of the coldest month	bio6
Temperature annual range	bio7
Mean temperature of the wettest quarter	bio8
Mean temperature of the driest quarter	bio9
Mean temperature of the warmest quarter	bio10
Mean temperature of coldest quarter	bio11
Annual precipitation	bio12
Precipitation of the wettest month	bio13
Precipitation of the driest month	bio14
Precipitation seasonality	bio15
Precipitation of the wettest quarter	bio16
Precipitation of the driest quarter	bio17
Precipitation of the warmest quarter	bio18
Precipitation of the coldest quarter	bio19

Appendix S2 – Species distribution modelling

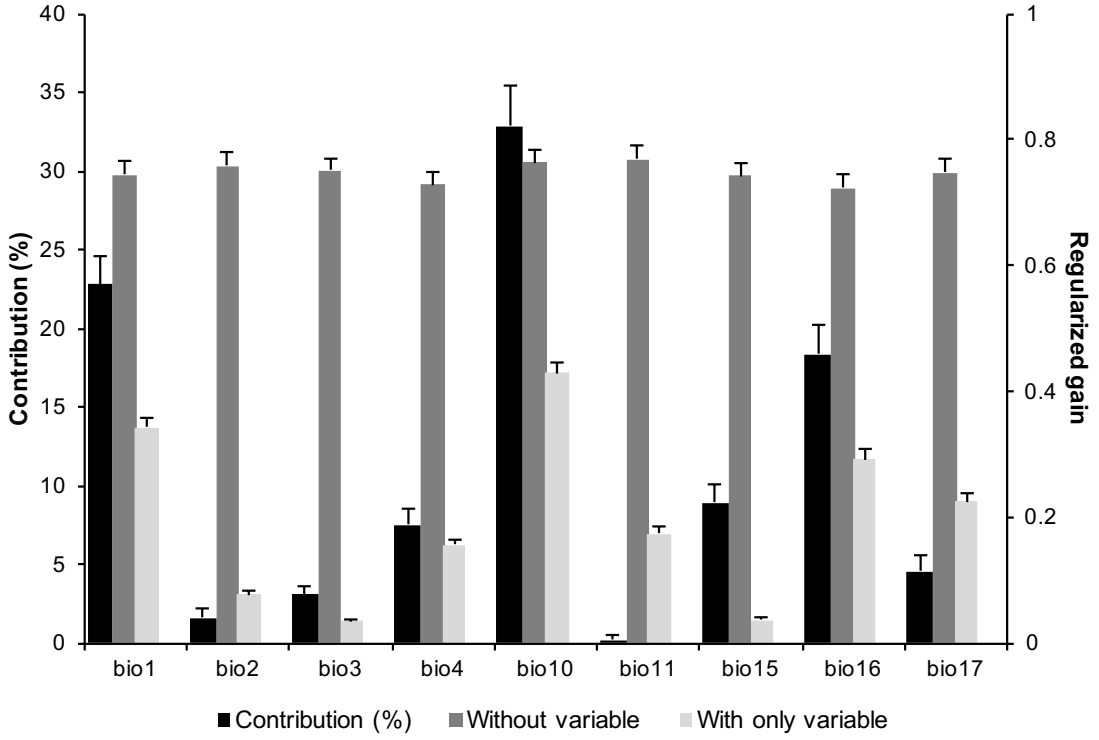

Fig. S2.1 – Graph representing variable importance for the *Plecotus auritus begognae* bioclimatic distribution model. The contribution, in percentage, of each variable is represented by the black bars, whose values can be read in the left axis of each plot. Dark grey bars represent the values of the jackknife results for models without the variable and light grey bars represent the same results for models with only one variable, these values can be read in the right axis of each plot. Variable abbreviations are available in Table S1.1.

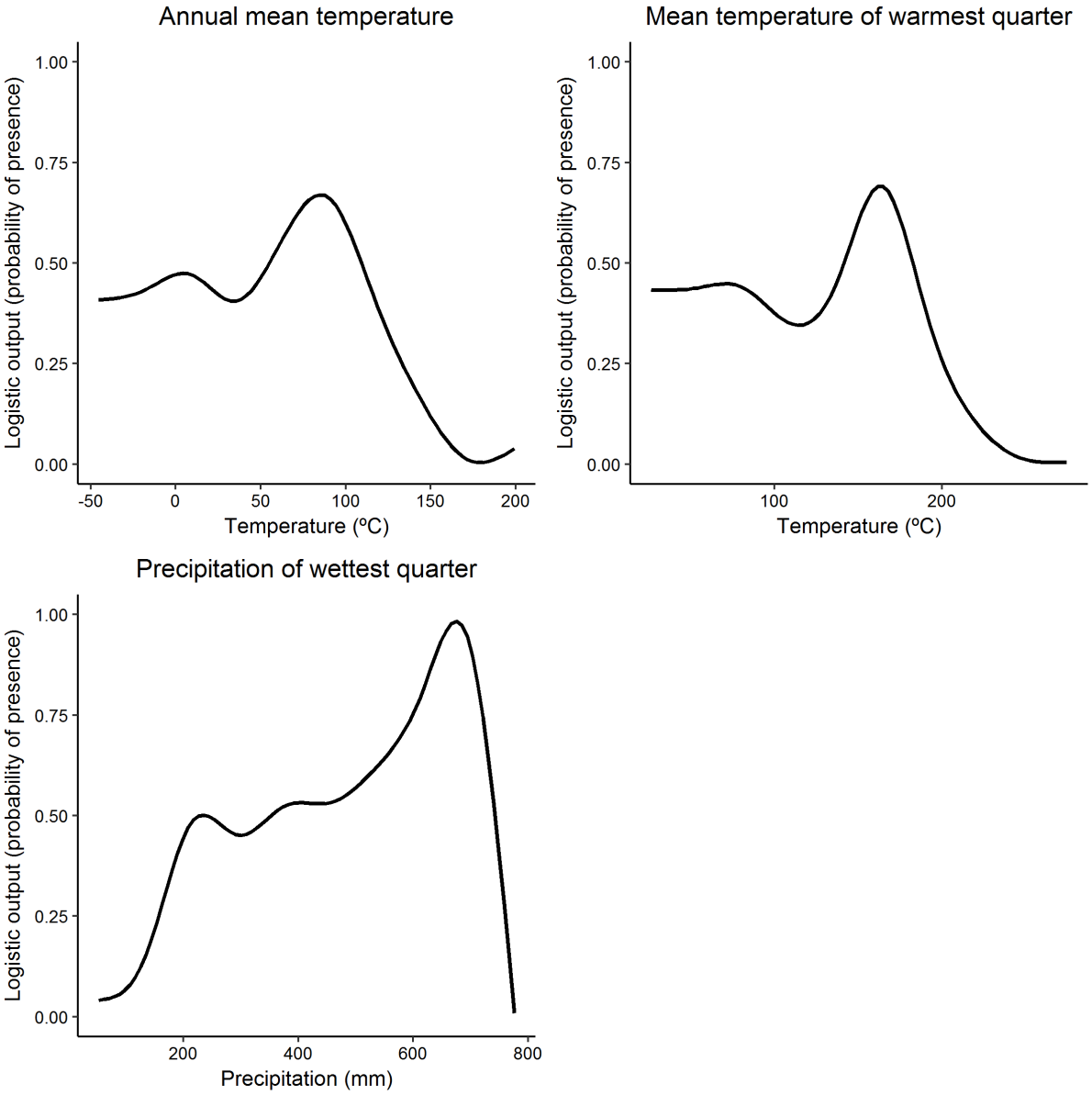

Fig. S2.2 – Response curves of the bioclimatic variables most related to the distribution of *Plecotus auritus begognae*.

CHAPTER 6

GENERAL DISCUSSION

1. NOVEL APPROACHES FOR BIODIVERSITY CONSERVATION

The development of novel methodologies that allow for fast and accurate development of conservation plans have become essential as a response to the increasing requirements in biodiversity conservation and management. This is particularly relevant for new and cryptic species, which are still being discovered (Bickford *et al.*, 2006; Sattler *et al.*, 2007). With the increasing development of faster and affordable molecular techniques, and more accurate spatial data and methodologies, there is a significant interest in the application of these tools to develop species' conservation measures. The combination of both molecular and spatial analyses, presents itself as an effective and approachable way to further inform on species conservation status, by allowing the assessment of its distributional range, ecological requirements, population structure, connectivity patterns and identification of populations under threat.

2. CONSERVATION OF BAT CRYPTIC SPECIES

2.1 A fast approach to survey bat cryptic species

With the recent advent of accessible molecular techniques, several new cryptic complexes have recently been found in bat species, particularly in Europe (Mayer & von Helversen, 2001; Ibáñez *et al.*, 2006; Mayer *et al.*, 2007). Consequently, there is a rising need for tools to rapidly assess their evolutionary, ecologic and conservation status. In this work, I intended to clarify these features for the cryptic bat species in the Iberian Peninsula. Although several studies have already been focusing on the molecular and evolutionary aspects of these complexes (Agirre-Mendi *et al.*, 2004; Juste *et al.*, 2004; Ibáñez *et al.*, 2006; García-Mudarra *et al.*, 2009; Salicini *et al.*, 2011; Juste *et al.*, 2013; Salicini *et al.*, 2013; Razgour, 2015), there is still little knowledge regarding their ecological and habitat requirements.

Already available sampling data for the cryptic bat species in the Iberian Peninsula, obtained in collaboration with several Iberian institutions, was used in the development of the first two chapters of this thesis. With these data, in a first approach, species distribution modelling techniques were implemented to infer ecological relationships between cryptic complexes and inform on species' possible distributional range and biogeographical affinities. By solely using this approach, the outputs were able to effectively inform on the probable occurrence range and environmental and habitat preferences of poorly-known cryptic species. Moreover, since

the focus of the work that followed was on the least studied cryptic complex, *Plecotus auritus auritus*/*Plecotus auritus begognae*, the results obtained allowed us to confirm the species' association with forested and mountainous areas, aiding in the sampling design and survey planning that followed. By identifying the species' possible areas of occurrence, a more efficient field campaign was successfully conducted, with the goal of filling the sampling gaps. Over the course of two years, data on the whole Iberian distributional range of *P. a. begognae* was obtained, allowing for a more comprehensive dataset, covering the whole range of the subspecies' environmental requirements.

2.2 Evolutionary history of *Plecotus auritus begognae*

Although several studies have focused on the possibility of an Iberian cryptic subspecies of the European Brown long-eared bat, *Plecotus auritus* (de Paz, 1994; Juste *et al.*, 2004; Ibáñez *et al.*, 2006), the low sampling size and the use of solely morphometry and single mitochondrial markers fuelled the need for further research on the lineage's evolutionary history in Iberia. The multi-marker phylogenetic study conducted allowed us to confirm that this Iberian lineage of *P. a. auritus* was probably formed after isolation within Iberia during the glacial period, differentiating itself from the European populations of this taxon. These analyses not only allowed the identification of P. a. begognae as an evolutionary significant unit, relevant for conservation management, but also the confirmation of its status a subspecies. The recognition of *P. a. begognae* as a different lineage was of extreme relevance for the subsequently conducted spatial analyses. Outputs obtained when modelling a lineage's distributional range, rather than the whole species', have shown to be more reliable and have higher resolution (Alberdi *et al.*, 2014).

The attainment of samples from the whole distributional range of *P. a. begognae*, and its recognition as a separate lineage of *P. a. auritus*, allowed for additional support when conducting the succeeding spatial analyses, which were of high data resolution demand (e.g. landscape genetics and connectivity analyses).

2.3 Extending the applicability of landscape genetics

The presented multi-disciplinary framework, integrating species distribution modelling with landscape genetics and spatially explicit connectivity methodologies, allowed for a better understanding of *P. a. begognae*'s population structure and movement patterns in the present and in future climate change scenarios. This approach was also revealed as a powerful tool

for the prioritization of areas for conservation, demonstrating which areas of the subspecies' distribution range are at risk, either of isolation or of contraction in future climate change scenarios. By merging these methodologies, a comprehensive spatial analysis of species' ecologic, genetic and connectivity dynamics, was obtained, while also identifying high priority populations (e.g. source-populations, at-risk populations) and areas of significance for conservation.

By solely using a landscape genetics approach, the applicability to conservation planning would be less evident, particularly since molecular studies are not always interpretable by conservation practitioners and policy-makers (Hoban *et al.*, 2013; Keller *et al.*, 2015). Moreover, even though they demonstrate the relevant gene flow movement routes, landscape genetics' studies do not present a quantitative way to prioritize the possible pathways, as such, conservation plans would have to focus on all the possible connectivity routes, which would be unfeasible. Having quantitative knowledge on species connectivity, allows for the identification and, therefore, prioritization, of the fragmentation-vulnerable areas and populations within the species' range. As for climate change studies, the majority seems to only focus on applying climate modelling to identify future suitable areas, without consideration of dispersal or gene flow (Bateman *et al.*, 2013). The integration of landscape genetics' analyses allows for a better understanding of the possible connectivity pathways to the predicted future suitable areas for the species, as well as the identification of which predictors are actually more likely to influence genetic variability in future climate change scenarios (Razgour *et al.*, 2013; Razgour, 2015). A deeper analyses of future species occurrence patterns, coupled together with landscape genetics and connectivity outputs, allows us to pinpoint the areas of high importance in the future, such as those with stable suitability, informing on present conservation plans to focus on the maintenance of these areas.

3. IMPLICATIONS FOR CONSERVATION

The outputs of this thesis may be used to provide detailed guidelines for the conservation of the Iberian population of the Brown long-eared bat. As a subspecies, *P. a. begognae* does not require an official conservation status and, as such, it holds the conservation status of *P. auritus*. Furthermore, when analysing its population structure, the lineage seems to be connected throughout its entire distribution range. Consequently, immediate conservation measures do not need to be undertaken for this population. However, the results outlined in the last two chapters, demonstrated possible areas at risk of fragmentation and of complete suitable habitat loss in future climate change scenarios. The patches which are most

vulnerable to isolation seem to be located in the most south-eastern region of the subspecies' distribution range, near the Iberian Range in the basin of the Ebro river. The smaller patches located in the most north-western region of the subspecies' distribution range, in western Galicia, even though located closer to the source population in northern Iberia, may also be at risk of isolation due to the existence of small habitat patches that are weakly connected to the remaining distribution. Moreover, when considering climate change projections, there seems to be a severe contraction of the subspecies' available suitable habitat. Most of the predicted subspecies' occurrence range will no longer be suitable by 2050, and almost entirely disappears by 2070. The patches located in the southern limit of the subspecies' distribution, in central Iberia, will become mostly unsuitable, with the exception of the mountain range of Serra da Estrela, located to the west, which seems to be one of the possible areas of refuge for the subspecies. Even though there seems to be a higher density of movement routes in the north of Iberia, north-eastern distribution patches, in the southern Pyrenees area, seem to also be in danger of becoming unsuitable. Most of the areas which seem to remain stable throughout the projections are located in north-western Iberia, in Galicia and the Cantabrian Mountains. Movement routes of the subspecies' will mostly be located in these regions, however, the routes located in the southern and eastern regions of the subspecies' distribution are of utmost importance to connect these populations to the stable areas in the north and west.

Future conservation efforts for the maintenance of the subspecies' population are to be directed to preserve connectivity between the regions in the Iberian Range and Ebro river basin, and the Galician and Cantabrian mountain regions. Forest continuity is of utmost importance to avoid the fragmentation of this population, as such, it should be maintained particularly in the southern and north-eastern distribution limits of the species, to assure population viability in future climate change scenarios. Forest patches throughout the north of the Iberian Peninsula become, therefore, of increasing relevance in maintaining population connectivity, serving as stepping-stones between suitable habitat areas (Saura *et al.*, 2014).

4. FINAL REMARKS

In order to better understand the mechanisms shaping *P. a. begognae*'s population structure, its connectivity and dispersal dynamics, and possible responses to climate change, further information on the subspecies' molecular characterisation is needed. The lack of population structure in this subspecies could be shaped by swarming mechanisms or female philopatry, where males display high dispersal behaviour and, therefore, diminish genetic variability

between colony areas (Veith *et al.*, 2004; Rivers *et al.*, 2005). More detailed information on the contribution of each sex for dispersal is necessary, and on possible locations of maternity colonies. Moreover, it would be of extreme interest to understand the evolutionary history of *P. a. begognae*, as a possible occurring speciation. A deeper knowledge of the ecological and molecular relationships between *P. a. begognae* and *P. a. auritus* would enlighten possible speciation processes. This information would also allow us to study potential hybridization mechanisms occurring between the two lineages. Of particular interest is the possible phenomenon of adaptive introgression, a feature that could ensure the survival and viability of populations (Hedrick, 2013). Moreover, recent studies on landscape genomics have proven successful in identifying species' adaptive capability to threats, such as climate change (Razgour *et al.*, 2017). This would aid in clarifying population's responses to climate change, informing on species' capacity to adapt or shift their ranges.

The greatest challenge to spatial analyses is the quality of data, rather than the suitability of algorithms. Ecogeographical variables with higher resolutions would allow for higher precision in calculating species' ecological niches and possible suitable habitat areas (Guisan *et al.*, 2007; Seo *et al.*, 2009). There's an increasing necessity for freely-available and better quality data to input in spatial methodologies, for the provision of more accurate results. The amount of species records also affects the possible outputs of spatial analyses (Wisz *et al.*, 2008; Bean *et al.*, 2012), and, therefore, efforts to increase sampling size for newly described cryptic species should be made.

Moreover, if *P. a. begonae*'s population history and possible distribution patterns in the Last Glacial Maximum were to be further analysed, landscape genetics and connectivity analyses could be combined in an attempt to understand the subspecies' past population movements to their current location. By comprehending these movement patterns, dispersal friction for colonizing new future areas could be calculated. These analyses would be able to improve the accuracy when modelling species' genetic diversity and current population structure, and give insights on possible population viability.

In conclusion, the work presented in this thesis presents a valid contribution in increasing the applicability of landscape genetics studies for conservation management. The integration of molecular analyses with spatial methodologies allowed the attainment of both ecological and genetic information of poorly-known species, while also presenting spatially explicit information essential to prioritize conservation measures. The use of both nuclear and mtDNA markers, together with microsatellite information, proved essential in clarifying species'

taxonomic status, and is therefore recommended in future descriptions of possible cryptic species, avoiding under-, or even overestimations, of species' diversity. The use of species distribution modelling to inform field surveys proved successful, as it already had in other studies for rare bat species (e. g. Rebelo & Jones, 2010). Molecular tools proved essential for establishing population structure, while also determining the environmental drivers of genetic differentiation. Coupled together with the environmental information provided by species distribution modelling outputs, this allows us to better understand the ecological and landscape factor shaping species distribution and gene flow patterns. By adding connectivity analyses, the determination of vulnerable areas within the species range was possible. The identification of at-risk populations and the main possible connectivity routes is essential to inform on conservation measures. Furthermore, by adding climate change modelling to the presented framework, the final outputs were able to demonstrate the possible future threats for the subspecies. It not only allowed the identification of the possible stable areas for population maintenance, but also the determination of the most likely dispersal routes for range shifting. All the methodologies and frameworks presented in this thesis may be applied to other taxa an in other geographical contexts, allowing for the more efficient design of pre-emptive conservation measures.

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APPENDIX A

PAPER PROOFS

INFLUENCES OF ECOLOGY AND BIOGEOGRAPHY ON SHAPING THE DISTRIBUTIONS OF CRYPTIC SPECIES: THREE BAT TALES IN IBERIA

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Influences of ecology and biogeography on shaping the distributions of cryptic species: three bat tales in Iberia

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To determine what shapes the distributions of cryptic species, we aimed to unravel ecological niches and geographical distributions of three cryptic bat species complexes in Iberia, *Plecotus auritus/begognae*, *Myotis mystacinus/alcathoe* and *Eptesicus serotinus/isabellinus* (with 44, 69, 66, 27, 121 and 216 records, respectively), considering ecological interactions and biogeographical patterns. Species distribution models (SDMs) were built using a presence-only technique (Maxent), incorporating genetically identified species records with environmental variables (climate, habitat, topography). The most relevant variables for each species' distribution and respective response curves were then determined. SDMs for each species were overlapped to assess the contact zones within each complex. Niche analyses were performed using niche metrics and spatial principal component analyses to study niche overlap and breadth. The *Plecotus* complex showed a parapatric distribution, although having similar biogeographical affinities (Eurosiberian), possibly explained by competitive exclusion. The *Myotis* complex also showed Eurosiberian affinities, with high overlap between niches and distribution, suggesting resource partitioning between species. Finally, *E. serotinus* was associated with Eurosiberian areas, while *E. isabellinus* occurred in Mediterranean areas, suggesting possible competition in their restricted contact zone. This study highlights the relevance of considering potential ecological interactions between similarly ecological species when assessing species distributions. *Biological Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: chiroptera – cryptic species – ecological niche – maximum entropy modelling – spatial PCA – species distributions.

INTRODUCTION

Recent concerns regarding biodiversity loss call for a deeper understanding of current species distributions patterns and how those patterns are shaped. This knowledge is highly relevant when accounting for species conservation planning and fundamental in ecological and evolutionary studies (Jetz, McPherson & Guralnick, 2012). Cryptic species, defined as ecologically and/or genetically distinct species with very

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similar morphology (Jones, 1997), pose a challenge to understanding species distributions as they bring the need to redefine what was once thought to be a single species' distribution and environmental requirements. Moreover, cryptic species may lead to biodiversity underestimates and may comprise threatened taxa within the cryptic complex that consequently require new conservation statuses (Bickford *et al.*, 2006; Sattler *et al.*, 2007).

Due to increasingly rapid DNA sequencing and the advances in molecular phylogenetic methods over the past decades, many cryptic species have recently been identified (e.g. Chadès *et al.*, 2008; Kaliontzopoulou *et al.*, 2011; Boratynski, Brito & Mappes, 2012). Indeed, one of the most diverse mammal groups in Europe – bats – has significantly increased in species number (Mayer & Helversen, 2001; Ibáñez *et al.*, 2006). In this context, the Iberian Peninsula possesses rich genetic diversity, mainly due to it being a major glacial refugium for several species (Hewitt, 2000; Gómez & Lunt, 2006; Razgour *et al.*, 2013), making the Mediterranean basin an important biodiversity hotspot (Myers *et al.*, 2000). Recent evidence suggests that 20% of Iberian bat species, which comprise nearly half of Iberia's mammal species (Mitchell-Jones *et al.*, 1999), harbour complexes of cryptic species (Juste *et al.*, 2004; Ibáñez *et al.*, 2006). There are six recently discovered cryptic bat species complexes in the Iberian Peninsula: *Plecotus auritus* (Linnaeus, 1758)/*begognae* (de Paz, 1994)/*macrobullaris* (Kuzyakin, 1965), *Myotis mystacinus* (Kuhl, 1817)/*alcathoe* (Helversen & Heller, 2001), *Eptesicus serotinus* (Schreber, 1774)/*isabellinus* (Temminck, 1840), *Myotis escalerai* (Cabrera, 1904)/sp.1, *Hypsugo savii*'s (Bonaparte, 1837) complex and *Pipistrellus kuhlii*'s (Kuhl, 1817) complex (Agirre-Mendi *et al.*, 2004; Ibáñez *et al.*, 2006). Despite the discovery of genetic discontinuities in these complexes, only *P. auritus/begognae/macrobullaris*, *M. mystacinus/alcathoe*, *E. serotinus/isabellinus* and *M. escalerai*/sp.1 exhibit high nucleotide divergence between the lineages (mtDNA distance over 8%) (Ibáñez *et al.*, 2006), indicating that these taxa may have experienced isolation during glacial periods (Pavan *et al.*, 2011). The *M. escalerai*/sp.1 complex has already been the focus of other studies (Salicini, Ibáñez & Juste, 2013) and we had very few data for *P. macrobullaris* so this species will not be considered in this study. Although these studies have provided genetic information, there is currently no information about these species' distributions or their ecological requirements in Iberia, or how these species are able to coexist.

The geographical distribution of a species is delineated by its ecological properties, namely the environmental conditions favourable for its occurrence, species interactions and dispersal capacity (Peterson, 2011). Environmental conditions (such as climate, habitat and availability of resources) will shape species distributions according to the geographical space where they meet species requirements for survival. Interactions with other species, either negative (competition, predation) or positive (facilitation), will further limit species distributions. All these factors will define the currently occupied distribution of a species, reflecting the accessible geographical space in which both biotic and abiotic conditions allow species existence (Peterson, 2011).

The use of species distribution models (SDMs) minimizes this caveat, by determining species potential distributions along with their environmental requirements. SDMs have been used effectively in several studies, aiding in the unravelling of the distributions of rare and cryptic species (Pearson *et al.*, 2007; Pineda & Lobo, 2009; Williams *et al.*, 2009; Rebelo & Jones, 2010; Rutishauser *et al.*, 2012; Bosso *et al.*, 2013). The data used in SDMs should enclose all the conditions explored by the studied species (Wisz *et al.*, 2008) although frequently disregard biotic interactions. However, it is recognized that interactions such as competition, predation or parasitism may restrict the environments in which a species may live, forcing it to occupy a narrower set of conditions (Hutchinson, 1957; Soberón & Peterson, 2005). Competition can have relevant ecological effects on the niches of species and, if these interactions are strong and pervasive enough, they may produce an evolutionary response in species' populations, for example, by competitive exclusion (Anderson, Peterson & Gómez-Laverde, 2002). However, competition can lead to a specialization of the resources explored by each species, allowing stable coexistence through time by species-specific differentiation in resource utilization (Hutchinson, 1978; Wang, Zhang & Wang, 2005). Trophic resource partitioning is known to occur in several cryptic bat species in Europe, including *Myotis myotis* and *M. blythii* (Arlettaz, Perrin & Hausser, 1997; Furman *et al.*, 2013), and habitat resource partitioning occurs in *Pipistrellus pipistrellus* and *P. pygmaeus* (Nicholls & Racey, 2006). These species coexist in sympatry, although they exploit different resources, such as prey, in the former case, or microhabitats, in the latter case.

The main purpose of this paper was to understand what shapes the distributions of cryptic species of bats in Iberia, and whether species share the same ecological background, thus developing hypotheses for current ecological interactions between cryptic species. Focusing on the cryptic complexes *P. auritus/begognae*, *M. mystacinus/alcathoe* and *E. serotinus/isabellinus* as case studies, the main questions addressed in this study were: (1) What is the spatial pattern of bat cryptic diversity in Iberia? (2) Which

ecological factors limit those patterns? (3) Do cryptic species share the same ecological conditions, i.e. are limiting factors similar? (4) Do biogeographical affinities shape these species' distributions and contact zones in Iberia?

MATERIAL AND METHODS

Study area

The study area was the Iberian Peninsula and the Balearic Islands, located in Europe's south-western extremity (Fig. 1A). It covers nearly 600 000 km^2 and it is bordered to the south and east by the Mediterranean Sea and to the north and west by the Atlantic Ocean, being divided from the rest of Europe by the Pyrenees mountain range in the north-east. It has a very heterogeneous topography and is roughly characterized by two main biogeographical regions: Eurosiberian and Mediterranean (Sillero *et al.*, 2009; Romo & García-Barros, 2010).

Sample collection

Distribution data for *Plecotus auritus/begognae*, *Myotis mystacinus/alcathoe* and *Eptesicus serotinus/isabellinus* were obtained from mist netting and roost trapping sessions in the Iberian Peninsula over the last decade (Ibáñez *et al.*, 2006; García-Mudarra, Ibáñez & Juste, 2009; Salicini, Ibáñez & Juste, 2011; Rebelo *et al.*, 2012; Salicini *et al.*, 2013). For each specimen, a tissue sample was collected in the field, through a small biopsy punch in the wing membrane. Afterwards, species identification for all records was validated by molecular analyses, following the procedure described by Ibáñez *et al.* (2006) (see Appendix S1, Supporting Information). Using phylogenetic analyses, we also compared sequences from samples collected for this study with those published in the above-mentioned studies (Appendix S2).

Prior to model calculations, spatial autocorrelation analyses were performed until data for each species achieved independence. For this, we used average nearest-neighbour analyses to remove clusters in the species' data. Consequently, for modelling there were 121 presence records for *E. serotinus*, 216 for *E. isabellinus*, 44 for *P. auritus*, 69 for *P. begognae*, 66 for *M. mystacinus* and 27 for *M. alcathoe*.

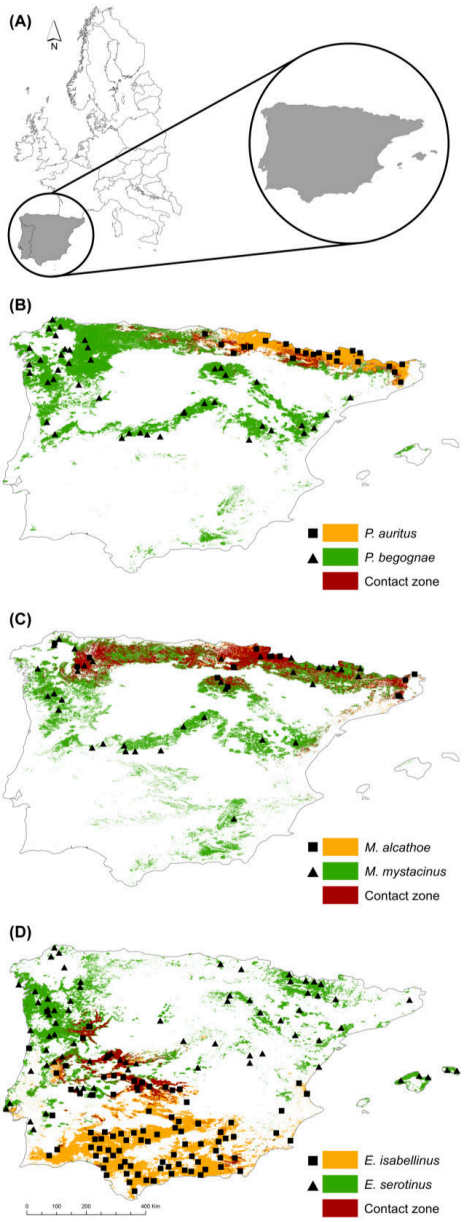

Figure 1. The study area in the European context (A). Potential distribution and presence records of the (B) *Plecotus auritus/begognae* complex, (C) *Myotis mystacinus/alcathoe* complex and (D) *Eptesicus serotinus/isabellinus* complex. Each symbol may correspond to more than one location.

ECOGEOGRAPHICAL VARIABLES

An initial set of 45 ecogeographical variables (EGVs) were chosen as predictors (see Table S3, Appendix S3). The chosen set of EGVs aimed to represent the environmental predictors related to bat occurrence (Ulrich, Sachanowicz & Michalak, 2007). Climate conditions are related to bat physiology, energy demands and water availability (Racey, Speakman & Swift, 1987; Webb, Speakman & Racey, 1995; Adams & Hayes, 2008; Frick, Reynolds & Kunz, 2010). Land cover, and distances to different habitat classes and to slopes are associated with potential foraging areas and roosts for tree- and crevice-dwelling bats (Russo & Jones, 2003; Rainho & Palmeirim, 2011). Climatic variables were obtained from WorldClim (http://www.worldclim.org), topographical variables were obtained from the digital elevation data of the NASA Shuttle Radar Topography Mission (http://srtm.csi.cgiar.org) and habitat variables were obtained from the Globcover project (http://postel.obs-mip.fr/?GLOBCOVER-Project) and from 'Mapa Forestal de España' (Garcia Viñas *et al.*, 2008) for the eucalyptus data.

All variables had a resolution of 300 × 300 m and respective calculations were made in ArcGIS 10.0 (ESRI, 2010).

SPECIES DISTRIBUTION MODELLING

SDMs were built using the maximum entropy modelling technique, Maxent version 3.3.3k (Phillips, Anderson & Schapire, 2006). Due to its reliability when using presence-only data, this technique has proven to outperform other modelling methods even with limited datasets (Hernandez *et al.*, 2006; Wisz *et al.*, 2008; Elith, Kearney & Phillips, 2010; Rebelo & Jones, 2010). Bats are known for their elusive and nocturnal behaviour (Ahlén & Baagoe, 1999), thus presenting limited detectability and identification in flight. The use of presence-only data in our study aims to overcome the issue of 'false absences', referring to situations when a species was not detected although it was present (Elith *et al.*, 2010).

In a first approach, species presence records, as the dependent variables, and the selected EGVs, as the independent variables, were imported into Maxent and run in auto features with a regularization multiplier of 2. The regularization multiplier was selected after model selection tests calculated trough ENM Tools 1.3 (Warren, Glor & Turelli, 2010) (http://enmtools.blogspot.com) and chosen according to its Akaike information criterion corrected for small sample sizes (AICc) value. Afterwards, 20 model replicates were run using cross-validation, in which the whole presence data set is randomly split into equal-sized partitions. The area under the curve (AUC) of the receiver operating characteristics (ROCs) plot was taken as a measure of the overall fit of the models (Fielding & Bell, 1997). The AUC ranges from 0, complete randomness, to 1, perfect discrimination (Phillips *et al.*, 2006).

To decrease the number of variables for the final distribution models, we first eliminated the highly correlated variables by first calculating a correlation matrix and then selecting the pairs of variables with correlations above 0.80 (Elith *et al.*, 2010). From these correlated groups of variables, only the most relevant variables for all species were considered, by using the percentage contribution values and the jackknife values of regularized training gain (a measure of likelihood between species presence data and the variable) obtained in Maxent for each species (Elith *et al.*, 2011).

A selected set of 15 variables (Table 1) was then used to build the final models, which we ran using the same settings as the initial models. Using these results, variable contribution plots and univariate response curves for the four most important variables for each species were built in Statistica© software.

The SDMs built were then imported into ArcGIS 10.0 and reclassified into presence–absence using the maximum training sensitivity plus specificity logistic threshold value (Liu, White & Newell, 2013). The reclassified models were then overlapped to define the possible contact zones between the species within each cryptic complex.

NICHE ANALYSES

Using the final SDMs calculated we proceeded to analyse the ecological niches of each species. Niche overlap (Warren, Glor & Turelli, 2008) and niche breadth (Nakazato, Warren & Moyle, 2010) statistics for each species were calculated using ENMTools 1.3. Niche overlap analyses were applied within each cryptic complex and considered the values of three indexes, Schoener's D (Schoener, 1968), the I statistic (Warren *et al.*, 2008) and relative rank (Warren & Seifert, 2011). Also, Levin's index (Levins, 1968), to determine niche breadth, was calculated for each species. Spatial principal components analysis (sPCA) was used to visualize the ecological niches of the different species. Initially, we extracted the values of each EGV for each grid cell of our study area, then, using these values, a PCA was calculated in R version 2.15.3 (R Core Team, 2012). The resulting values of the PCA components were then imported into ArcGIS 10.0 and the values of each species presence records were plotted for each component. We calculated minimum convex polygons to delimit each species environmental space from the samples included in this study (hereafter termed the realized niche) and measured their individual areas and the area of overlap between species' niches.

Table 1. Set of variables used in the final species distribution models

Type	Variable	Code
Climatic	Annual mean temperature (°C)	ib_bio1
	Mean diurnal range (°C)	ib_bio2
	Mean temperature of warmest quarter (°C)	ib_bio10
	Mean temperature of coldest quarter (°C)	ib_bio11
	Precipitation of wettest quarter (mm)	ib_bio16
	Precipitation of driest quarter (mm)	ib_bio17
Topographical	Altitude (m)	ib_dem
	Maximum slope (°)	ib_slopemax
	Distance to slope > 20° (m)	ib_di_sl20
	Distance to maximum slope > 20°(m)	ib_di_slmax20
Habitat	Land cover:	ib_land
	Agriculture 1	
	Orchards 2	
	Forested agriculture 3	
	Forest 4	
	Coniferous 5	
	Shrubs 6	
	Bare 7	
	Urban 8	
	Water 9	
	Eucalyptus plantations 10	
	Distance to forests (m)	ib_di_forest
	Distance to agriculture (m)	ib_di_agric
	Distance to water bodies (m)	ib_di_water
	Distance to eucalyptus plantations (m)	ib_di_eucal

RESULTS

Important ecogeographical variables for species distributions

The variables that contributed the most to the distribution models built differed among species. Graphs of variable contributions and respective response curves can be found in Figures S3.1–S3.12 in Appendix S3. When analysing the response curves for these variables we can see that both *P. auritus* and *P. begognae* are more likely to occur at low temperatures and in areas with high precipitation. *Plecotus begognae* also favoured the presence of steep slopes. Low temperatures and proximity to steep slopes seem to favour the occurrence of *M. mystacinus*. The response curves of *M. alcathoe* showed that this species' occurrence is more likely in areas with high precipitation and in close proximity to forests and to steep slopes. In the case of *E. serotinus*, occurrence is most favoured at high precipitation and in close proximity to forests and steep slopes. Finally, *E. isabellinus* seems to have a higher probability of occurrence in areas with moderate temperatures, low precipitation and within and in the vicinity of eucalyptus plantations.

Species distributions and contact zones

The SDMs built exhibited ROC curves with high average AUCs, with all species presenting similar values, these being 0.98 ± 0.016, 0.91 ± 0.025, 0.91 ± 0.054, 0.96 ± 0.015, 0.86 ± 0.065 and 0.92 ± 0.034, for *P. auritus*, *P. begognae*, *M. mystacinus*, *M. alcathoe*, *E. serotinus* and *E. isabellinus*, respectively. In the case of the *P. auritus/begognae* complex (Fig. 1B), the models predicted the distribution of *P. auritus* to be restricted to the Pyrenees area and *P. begognae* to occur mostly through northern Iberia and the Balearic Islands with a patchy distribution in the central region mountains. Due to these focused distributions, the contact zone of these two species seems to be delimited by the low elevations of the Pyrenees. When considering the model's results for the *M. mystacinus/alcathoe* complex (Fig. 1C), it seems that both species have a northern distribution in Iberia, with *M. mystacinus*' distribution extending further south than *M. alcathoe*'s. Also, *M. alcathoe*'s distribution is mostly contained within the wider distribution of *M. mystacinus*. Finally, the models demonstrated a high degree of separation for *E. serotinus/isabellinus* (Fig. 1D) in Iberia. Although somewhat patchy, the distribution of *E. serotinus* was mainly concentrated in

Table 2. Niche overlap statistics

Schoener's *D*		*I* statistic		Relative rank	
	P. begognae		*P. begognae*		*P. begognae*
P. auritus	0.11	*P. auritus*	0.26	*P. auritus*	0.77
	M. mystacinus		*M. mystacinus*		*M. mystacinus*
M. alcathoe	0.48	*M. alcathoe*	0.77	*M. alcathoe*	0.82
	E. serotinus		*E. serotinus*		*E. serotinus*
E. isabellinus	0.29	*E. isabellinus*	0.54	*E. isabellinus*	0.50

Table 3. Niche breath (Levin's index)

	Inverse concentration	Uncertainty
P. auritus	0.01	0.77
P. begognae	0.12	0.90
M. mystacinus	0.12	0.90
M. alcathoe	0.05	0.86
E. serotinus	0.22	0.95
E. isabellinus	0.10	0.91

Table 4. Overlap values of sPCA analyses

	Realized niche		
Species	Area	Overlap (%)	Area in Iberia (%)
P. auritus	8.54	25.64	8.45
P. begognae	11.85	18.48	11.72
M. mystacinus	18.06	22.81	17.87
M. alcathoe	4.12	100.00	4.08
E. serotinus	30.07	35.42	29.75
E. isabellinus	20.86	51.05	20.64

the north of Iberia and in the Balearic Islands. The distribution of *E. isabellinus* is focused in the south of Iberia, and it is not predicted to occur in the Balearic Islands. Accordingly, the contact zone between these two species' distributions appears to be in the central regions of Iberia.

NICHE ANALYSES

For the analyses of niche overlap and niche breadth using the distribution models calculated for each species, we present the overlap statistics for each cryptic complex (Table 2) and the niche breath values for each species individually (Table 3). Also, the sPCA built allowed us to determine the overlap between the occupied species' environmental niches in Iberia (Table 4). The sPCA obtained for the *P. auritus/begognae* complex (Fig. 2A) shows that both species have different and somewhat restricted niches within Iberia. *Plecotus begognae*'s niche (Area = 11.85) is slightly broader than that of *P. auritus* (Area = 8.54) and the overlap between both species' niches is similar (25.64% for *P. auritus* and 18.48% for *P. begognae*), with *P. auritus* having a slightly larger amount of its niche area within *P. begognae*'s niche (Table 4). In the case of the *M. mystacinus/alcathoe* complex (Fig. 2B), both species seem to occupy similar conditions in Iberia, although *M. alcathoe*'s niche area (Area = 4.12) is much smaller than *M. mystacinus*' (Area = 18.06). Moreover, *M. alcathoe*'s niche is completely within that predicted for *M. mystacinus*, while only 22.81% of the niche area of *M. mystacinus* is occupied by *M. alcathoe*'s niche. Lastly, in the *E. serotinus/isabellinus* complex (Fig. 2C), both species occur in a relatively large range of conditions in Iberia. *Eptesicus isabellinus* has half of its niche overlapping with *E. serotinus*, whilst *E. serotinus* only has 35.42% of its niche within that predicted for *E. isabellinus*.

When analysing the sPCA, we were able to distinguish two main biogeographical patterns with two strata, Eurosiberian and Mediterranean (Fig. 3). As for the biogeographical patterns of the contact zones between each complex, we can see contact zones for *P. auritus/begognae* (Fig. 3A) and *M. mystacinus/alcathoe* (Fig. 3B) overlapping in relatively the same biogeographical area located in the north of the Iberian Peninsula. As *M. mystacinus/alcathoe*'s contact zone is broader, it occurs to a small extent in central Iberia, but mainly follows the same biogeographical pattern, i.e. present in the north, avoiding the biogeographical strata of the south. As for the *E. serotinus/isabellinus* complex (Fig. 3C), the pattern is less clear. The contact zone between these species seems to be mainly located in transition areas between the two main biogeographical strata, but is mainly situated in the central–western area of Iberia.

DISCUSSION

This study reveals the potential distributions of newly discovered cryptic complexes of bats in the Iberian Peninsula and also, by analysing their niches indi-

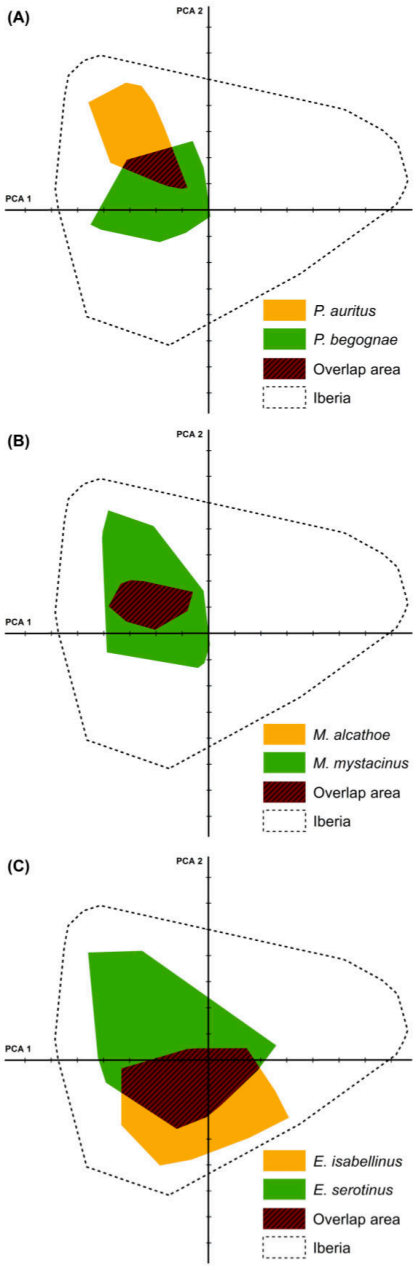

Figure 2. Spatial principal component analyses of the ecological niches of (A) the *Plecotus auritus/begognae* complex, (B) *Myotis mystacinus/alcathoe* complex and (C) *Eptesicus serotinus/isabellinus* in Iberia. Filled polygons represent the realized niche and the dotted line represents the environmental space available in Iberia.

vidually and within complexes, aids in the understanding of these species' ecological requirements and how they may be affecting each other's distributions. Different distribution patterns were observed within and between complexes: most species were concentrated in the north of Iberia (*P. auritus*, *P. begognae*, *M. mystacinus*, *M. alcathoe* and *E. serotinus*), and one species occupied southern Iberia (*E. isabellinus*). These distribution patterns clearly associate with the known biogeographical affinities of each species (Horáček, Hanák & Gaisler, 2000), where the majority have a clear Eurosiberian/Atlantic distribution and only *E. isabellinus* shows an association with Mediterranean areas. When analysing species' ecological niches, we observed that *P. auritus* and *M. alcathoe* had considerably narrower niches when compared with the other species, as supported by studies that reveal these species as specialists (Helversen *et al.*, 2001; Ashrafi *et al.*, 2011). *Eptesicus serotinus* had the broadest niche, which was expected as it is known to be a generalist species (Catto *et al.*, 1996). Strong niche dynamics occur within complexes, resulting from either an established equilibrium or other ecological interactions that occur (e.g. competition) and realized niches in Iberia may still be adjusting. Historical factors should also be considered. For example, it is possible that some European lineages (such as *P. auritus*) have recently arrived in Iberia and species distribution limits may still be under definition processes. In fact, each of the species complexes focused on in this study seems to have a unique story to tell.

THE *PLECOTUS* TALE

Both species distributions in the *Plecotus* complex seem to be highly related to climatic variables and to the presence of slopes. In fact, when analysing this complex's distribution patterns, *P. auritus* seems to mainly occur in the mountainous areas of the Pyrenees, while *P. begognae* occupies the rest of northern Iberia, notably excluding the Pyrenees. *Plecotus begognae*, a recently discovered lineage in the Iberian Peninsula, had a relatively unknown distribution, ecological requirements and biogeographical affinity. However, de Paz (1994) found this species in the central mountainous regions of Iberia, south of the Cantabrian Mountains and south of the Pyrenees, later confirmed by Ibáñez *et al.* (2006). Although both

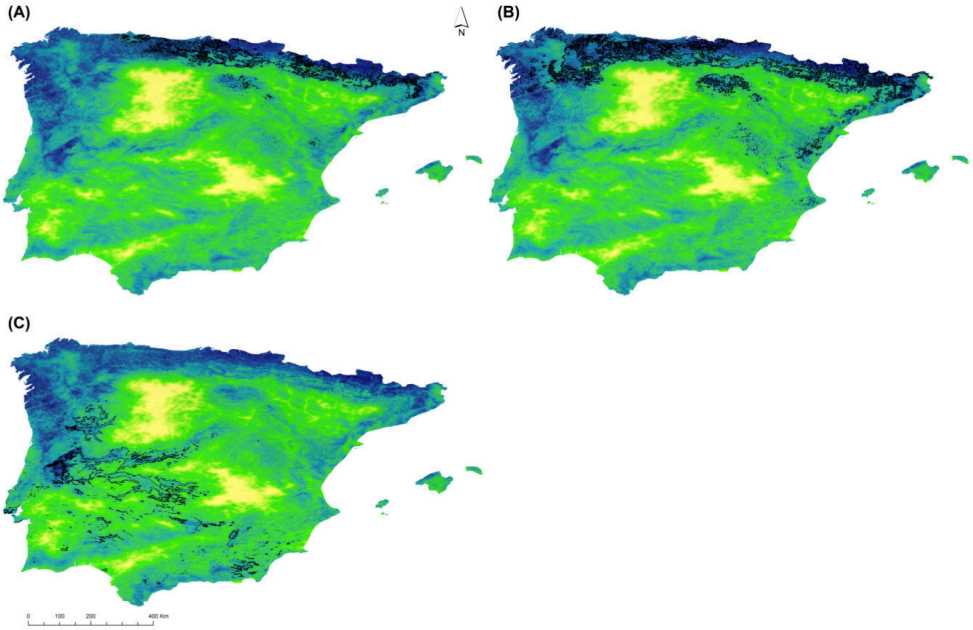

Figure 3. Spatial principal component analysis with the two main biogeographical strata identified for Iberia. The blue areas (darker tones) represent Eurosiberian areas and the green to yellowish areas (lighter tones) Mediterranean areas. The contact zones of (A) the *Plecotus auritus/begognae* complex, (B) *Myotis mystacinus/alcathoe* complex and (C) *Eptesicus serotinus/isabellinus* complex are delineated in black.

Plecotus species occupy the northern areas of Iberia and appear to have similar biogeographical affinities, their distributions have relatively little overlap restricted to a few areas located in north-eastern Iberia (south of the Pyrenees), reflected also in a small niche overlap. This complex's parapatric distribution could be due to resource competition, leading to competitive exclusion. Rutishauser *et al.* (2012) found parapatric distributions in two *Plecotus* species in Switzerland (*P. austriacus* and *P. macrobullaris*) probably resulting from competitive exclusion, supported by strong similarities in their echolocation signals, flight behaviour (Dietrich *et al.*, 2006) and diet (Ashrafi *et al.*, 2011). Other bat species have been known to have parapatric distributions due to competitive exclusion; for example, *Vespertilio murinus* is excluded by *Eptesicus serotinus* on some Danish islands, presumably as a consequence of competition (Baagøe, 1986) or *P. pipistrellus* and *P. kuhlii* in southern Switzerland, where *P. kuhlii* seems to restrict *P. pipistrellus* to the uplands as a result of competitive pressure (Haffner & Stutz, 1985). However, coexistence also occurs in *Plecotus* species; trophic niche partitioning exists between *P. auritus* and *P. macrobullaris*, and *P. auritus* and *P. austriacus* coexist probably by niche partitioning in Central Europe and the Alps (Ashrafi *et al.*, 2011). Therefore, only through small-scale studies (radiotracking, diet analyses, etc.) will it be possible to assess the interactions between *P. auritus* and *P. begognae*, and whether competition can potentially occur.

THE *MYOTIS* TALE

Myotis mystacinus and *M. alcathoe* are restricted to the north of Iberia, with *M. mystacinus* showing a relatively wider distribution than *M. alcathoe*. Accordingly, both species occur in the Eurosiberian part of Iberia, mainly in mountainous regions (Agirre-Mendi *et al.*, 2004), which explains the relevance of climatic and topographic variables in explaining the distributions of these species. Although these species have similar distributions to the *Plecotus* complex, the overlap between them

is rather different. *Myotis alcathoe*'s distribution is almost completely contained within the distribution of *M. mystacinus*, and its entire realized niche is within that of *M. mystacinus*. It appears that these species' distributions are sympatric and some sort of niche equilibrium is occurring between them. We suggest that stable coexistence may occur between these species, with a likely species-specific differentiation in resource utilization. Indeed, it is thought that *M. alcathoe* is sympatric with *M. mystacinus*, exploring mainly stands of deciduous trees near bodies of water (Helversen *et al*., 2001), explaining the relevance of distance to forests in this species' distribution models. However, *M. alcathoe* differs from the other European *Myotis* species due to its restricted habitat requirements (Lučan, Hanák & Horáček, 2009), hence suggesting that *M. alcathoe* and *M. mystacinus*, although with a highly sympatric distribution, are able to coexist, most likely by exploring different micro-habitats through resource partitioning. In fact, Helversen *et al*. (2001) found that, in Greece, *M. mystacinus* appears to occur in forested marginal habitats, generally hunting near large bodies of water, whereas *M. alcathoe* is found in dense woodland, hunting along small streams. Such resource partitioning is known to occur in other sympatric species of the same genus, such as between *M. myotis* and *M. blythii* (Arlettaz *et al*., 1997) and between *M. bechsteinii* and *M. naterreri* (Siemers & Swift, 2006), highlighting the existence of species-specific micro-habitat preferences.

The *Eptesicus* tale

The *Eptesicus* complex is distinct from the patterns seen in the previous two complexes. Both *Eptesicus* species have distinct distributions in Iberia, with *E. serotinus* mainly in the north and *E. isabellinus* in the south. *Eptesicus serotinus* is known to occur in temperate climates (Rebelo, Tarroso & Jones, 2010), and Ibáñez *et al*. (2006) have suggested its presence to be restricted to the northern part of Iberia, although it could potentially occupy other parts of Iberia given that in other countries it was also found in more xeric areas (Juste *et al*., 2013). As *E. isabellinus* is a recently discovered species in Iberia, little is known of its actual distribution. However, García-Mudarra *et al*. (2009) suggested that *E. isabellinus* occupied the southern part of Iberia in allopatry with *E. serotinus*. Our results indicate that *E. isabellinus* may have a strong Mediterranean affinity, as it is predicted to occur mainly in southern Iberia where the Mediterranean climate is dominant, which would explain the relevance of climatic variables in this species' distribution models. In fact, a strong connection between Iberian and North African populations of *E. isabellinus* is shown by Juste *et al*. (2009). However, the two *Eptesicus* species have extensive niche overlap, indicating that they explore very similar conditions, although geographically only moderate overlap occurs (with the main contact zone in central Iberia). This suggests that each species thrives in a specific biogeographical zone, *E. serotinus* in Atlantic climates and *E. isabellinus* in Mediterranean areas, but considering the large niche overlap it is quite possible that competition may occur if prey resources are limited in contact zones. In fact, it was shown by Brito *et al*. (2009) with North African canids of the genus *Vulpes* that species with different biogeographical affinities but with overlap in habitat requirements may be suffering from spatial exclusion through competition. Bilgin *et al*. (2008) demonstrated that two morphologically similar species of *Miniopterus* bats were separated by climatic affinities. Later, Furman *et al*. (2010) showed that the geographical distributions of these species were the result of historical and ongoing expansion events, and interlineage competition rather than the outcome of climatic preferences. As such, only through future phylogeographical analyses and studies directed to habitat use and resource selection will it be possible to clarify this situation.

Limitations and caveats

When considering genetic methodology, we are aware of the possible limitations or errors of sequencing only mitochondrial DNA (Zhang & Hewitt, 2003). However, our samples were sequenced using markers developed by Ibáñez *et al*. (2006) that have been tested numerous times, which guarantee the correct identification of the sampled individuals.

To develop adequate prediction using distribution models, it is advisable to have the best sampling coverage of each species' distribution (Jiménez-Valverde, Lobo & Hortal, 2008). Moreover, it is quite frequent that species distribution modelling studies highlight new areas of occurrence (e.g. Raxworthy *et al*., 2003; Rebelo & Jones, 2010). In this study, the long-term sampling effort over several years ensured almost complete coverage of the Iberian Peninsula (the exception being the north–central region of the Cantabrian mountain ridge in Spain). By comparing distribution models with the sampling coverage there was a strong indication that each species' realized distribution was covered. However, some isolated patches of suitability were predicted by the models, in extreme areas of species' distributions. These areas are known to be out of the distribution limits of the species; for example, *P. auritus* has not been found in the Balearics nor *M. mystacinus* in the mountains of Andalucia. These areas could have been beyond the dispersion capacity of the species.

Finally, when analysing possible ecological niches of the species and the interactions between them through ecological niche modelling, we must take into account the resolution of the variables and the scale of the analyses (Soberón & Nakamura, 2009). Biotic interactions such as competition or resource availability are generally studied at local scales and are barely considered at larger scales (Whittaker, Willis & Field, 2001). Thus, we propose only possible interactions that may occur between the studied species within each complex, according to realized niche as delimited by the resolution of our models. These are hypotheses that need further study at local scales within each of the contact zones for each species complex.

CONCLUSIONS

The analyses of species distributions and ecological niches allowed us to differentiate several possible interactions occurring within the cryptic complexes studied. We observed niche overlap between species occupying similar biogeographical regions (the *Plecotus* and *Myotis* complexes), and between species occupying different biogeographical regions (the *Eptesicus* complex). First, in the *Plecotus* complex distributions could be explained by competitive exclusion. The *Myotis* complex showed considerable overlap in niches and distributions between species, suggesting stable coexistence by resource partitioning. Finally, in the *Eptesicus* complex there is a higher uncertainty as to what is occurring, as there was a surprisingly significant overlap between both species' ecological niches, but a relatively small overlap in space, resulting in a few contact zones. This could be a phenomenon of competitive exclusion where the niches have stabilized, but their overlap can also imply that the species' niches are still evolving and that competition is actively occurring.

When analysing the biogeographical patterns of the contact zones, we see a clear association of the contact zones of the *Plecotus* and of the *Myotis* complexes to the Eurosiberian regions. The contact zone of the *Eptesicus* complex seems to be in the areas of transition between the Eurosiberian and Mediterranean biogeographical zones, which are associated with the affinities of *E. serotinus* and *E. isabellinus*, respectively.

This study brings relevant insight into the distributions and biogeographical affinities of cryptic species, allowing the determination of contact zones within complexes. It highlights the importance of how different interactions within complexes can influence each species' ecological niche and, consequently, its geographical distribution. Determining cryptic species' distributions and their ecological requirements and interactions will aid in conservation management and with establishment of the conservation status of the newly discovered species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Genetic methodology.
Appendix S2. Phylogenetic analyses.
Appendix S3. Ecogeographical variables and variable importance.

APPENDIX B

OTHER PAPERS ARISING FROM THE PHD RESEARCH

BATS' ECHOLOCATION CALL CHARACTERISTICS OF CRYPTIC IBERIAN *EPTESICUS* SPECIES

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ORIGINAL ARTICLE

Bats' echolocation call characteristics of cryptic Iberian *Eptesicus* species

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Abstract Advances in molecular methods and analyses, such as DNA sequencing and phylogenetic reconstructions, are being widely used to help clarify the taxonomic challenge posed by cryptic species. While almost morphologically indistinguishable, such species can also present other diagnostic features, including ecological and physiological characteristics. The main goal of this work was to investigate whether it is possible to distinguish two cryptic bat species in Iberia, *Eptesicus serotinus* and *Eptesicus isabellinus*, through their echolocation call characteristics. After molecular identification of species' colonies, echolocation calls were recorded during emergence from roosts for 52 individuals. A stepwise discriminant analysis was used to test if the variables measured in the recordings could significantly differentiate the two species. This analysis was able to extract one discriminant function, with the variables' peak frequency and duration of pulses identified as statistically significant. These provided a correct overall classification of approximately 78.8 %. We found that on average peak frequency is higher in the echolocation calls of *E. isabellinus* compared with that of *E. serotinus*, but overlap occurred between 23.4 and 28.8 kHz. Moreover, in our recording conditions, calls belonging to *E. isabellinus* tended to be shorter than those of *E. serotinus*. Possibly, some acoustic differences could be explained by local adaptations to different climate conditions and ecological niches experienced by each species.

Keywords Acoustic identification · Cryptic species · Echolocation calls · *Eptesicus isabellinus* · *Eptesicus serotinus* · Iberian Peninsula

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Introduction

The advent of relatively inexpensive and rapid DNA sequencing together with developments in molecular phylogenetic methods have allowed great advances in the detection and differentiation of morphologically similar taxa, clarifying the taxonomic challenges long posed by cryptic species (Bickford et al. 2007). Consequently, several cryptic bat species (ecologically and/or genetically distinct species with similar morphology (Jones 1997)) have recently been identified worldwide (e.g. Chadès et al. 2008; Kaliontzopoulou et al. 2011; Boratynski et al. 2012). However, in some cases, besides genetic differentiation, cryptic species may present other diagnostic features such as physical or ecological characteristics (Saez and Lozano 2005; Schlick-Steiner et al. 2007; Santos et al. 2014). Several bat species show differences in their echolocation calls, despite their morphological similarity (Brigham et al. 2002; Jones and Barlow 2004).

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Recently, the Iberian Peninsula has been in the focus of several studies resulting in the recent discoveries of a number of complexes of cryptic species (e.g. Pinho et al. 2007; Paupério et al. 2012). The existence of such complexes could be a consequence of the high genetic diversity in populations that resulted from Iberia being an important glacial refugium (Hewitt 2000; Gómez and Lunt 2006; Razgour et al. 2013). In fact, recent studies suggest that 20 % of the traditionally recognized bat species in the Iberian Peninsula comprise complexes of cryptic species (Juste et al. 2004; Ibañez et al. 2006). Of these, four species pairs exhibited high nucleotide divergence between lineages (Ibañez et al. 2006; Santos et al. 2014): *Plecotus auritus* (Linnaeus 1758)/*begognae* (de Paz 1994)/*macrobullaris* (Kuzyakin 1965), *Myotis mystacinus*/*alcathoe* (von Helversen et al. 2001), *Myotis escalerai* (Cabrera 1904)/sp.1. and *Eptesicus serotinus* (Schreber 1774)/*isabellinus* (Temminck 1840).

The latter exhibits a level of genetic divergence over 16 % (Ibañez et al. 2006). *E. serotinus* has a broad range covering most of Europe up to a latitude of 55° N, albeit with a fragmented distribution. In contrast, *E. isabellinus* seems to be restricted to southern Iberia and North Africa (Dietz et al. 2009; Juste et al. 2009; Santos et al. 2014).

The discovery of several cryptic species complexes in the last two decades raises the challenge of gathering knowledge about biological traits of the new species. The use of ultrasound survey techniques has been very prolific and valuable for the study of bats' ecology. Data collected from bat detectors have been used in a wide range of studies including the assessment of habitat use (e.g. Russo and Jones 2003), behaviour (e.g. Seibert et al. 2013), evolutionary patterns (e.g. Russo et al. 2007) and in environmental impact assessments (e.g. Weller and Baldwin 2013). Moreover, acoustic surveys frequently contribute to the development of conservation plans, guidelines or policies (Fenton 1997). Characterization of the vocalizations of cryptic species is important, especially as cryptic bat species often show niche separation (e.g. Davidson-Watts and Jones 2006). Moreover, acoustic surveys often allow rapid and easy separation of similar species that may otherwise be difficult to distinguish (Brigham et al. 2002). Regarding the aforementioned Iberian cryptic bat species, the *Eptesicus* species have the greatest potential to be identified through ultrasound analysis as their calls end with a narrowband component that differs in frequency in other cryptic taxa such as Pipistrellus (Walters et al. 2012).

The main purpose of this research was to understand if echolocation call characteristics differ between Iberian *Eptesicus* species (*E. isabellinus* and *E. serotinus*). The main questions addressed were the following: (1) what are the echolocation call characteristics of the Iberian *Eptesicus* species? (2) Is it possible to identify the *Eptesicus* species based on echolocation call data only?

Methods

Study area

This study was done in Portugal which includes areas of two main biogeographic regions: Eurosiberian and Mediterranean (Sillero et al. 2009; Romo and García-Barros 2010). The two *Eptesicus* species exhibit a parapatric distribution in Portugal with *E. serotinus* mainly occurring in the Eurosiberian areas of the north of Iberia while *E. isabellinus* is more associated with the Mediterranean south (Santos et al. 2014).

Recording of echolocation calls

Recordings were made during bat emergence from monospecific roosts (two roosts of both species) (*E. serotinus*, roost 1: N=16 individuals; roost 2: N=4 individuals; *E. isabellinus*, roost 1: N=19 individuals; roost 2: N=22 individuals). Distances between all roosts exceeded 150 km. Recordings were only analysed after molecular identification of bats (Santos et al. 2014). Sites presenting similarities in vegetation structure (at least the 300 m surrounding the roosts were dominated by open habitats: *E. serotinus*—agricultural landscapes; *E isabellinus*—pastures) were visited only once to avoid pseudo-replication (Hurlbert 1984). Calls were recorded only when bat linear trajectories were visible (100±50 m away from the roost exit) so that the usual steep broadband calls emitted immediately after emergence would not be included in our dataset. Bats were recorded flying alone in the open at least 10 m above ground in all situations. Recordings were made using a D-240X bat detector (Pettersson Elektronik AB, Uppsala). The resulting sample, lasting 17 s (with 10× time expansion), was then played back and digitally recorded with a Roland Edirol R-09 recorder (sampling rate of 44.1 kHz and files recorded in WAV format).

Sound analyses

Recordings were analysed with the software BatSound 4 (Pettersson Elektronik AB, Uppsala) using a 512-pt FFT with a Hamming window for spectrogram analysis. Call variables were measured using crosshair screen cursors. Four pulses were randomly selected from each recording for analysis. The following seven variables were measured in each pulse: maximum frequency (the highest frequency value measured; FMax); minimum frequency (the lowest frequency value measured; FMin); peak frequency (the frequency with maximum

energy; PeakF); frequency at the middle of the pulse (FMid_pulse); slope at the middle of the pulse (Slope_Mid_Pulse; slope was measured considering the frequencies ±0.5 ms from the middle of the pulse); duration of the pulse (Dur); inter-pulse interval (the time interval between two consecutive pulses, from the end of first pulse until the start of next; IPI). Dur and IPI (ms) were measured from oscillograms, PeakF (kHz) from power spectra, and all other spectral variables (kHz) from spectrograms. Measurements were taken from the first harmonic as this contained the most energy.

Statistical analyses

Medians of the subset of four pulses measured randomly in each individual echolocation call sequence were included in the dataset, resulting in a single value per individual for each acoustic variable (*E. serotinus* (N=18) and *E. isabellinus* (N=34)). These data were included in a stepwise discriminant analysis with the method of Wilks' Λ used to identify which of the variables under study can better discriminate the two species. FMax variable was initially eliminated because it was very variable and highly dependent on the quality of the recordings (recordings of more distant bats showed low values of FMax). Assumptions of normality and homogeneity of variance-covariance matrices of each species were tested with Kolmogorov-Smirnov test and the Box M test, respectively. Variables Dur and Slope_Mid_Pulse did not present a normal distribution (p<0.05) for *E. isabellinus* and *E. serotinus*, respectively, so we proceeded both transformation through the square root.

According to the Box M test, the assumption of homogeneity of variance-covariance matrices is only valid for a statistical test of α=0.01 (M=8.961, $F(3, 31281)$=2.838, p=0.037). Still, because the discriminant analysis is robust to violation assumptions when (*i*) the size of the smallest group (*E. serotinus*) is greater than the number of variables in the study and (*ii*) the means of the groups are not proportional to their variances (Stevens 1986), as it was the case we proceeded with the analysis. Finally, we used Classification Statistics to obtain the classification functions and to assign species identification for new echolocation calls. All analyses were carried out with α=0.05 (except the Box M test) using the software IBM SPSS Statistics (v. 22; SPSS Inc, Chicago, IL).

Results

Testing for differences between species

Echolocation calls were recorded from 52 individuals after roost emergence. PeakF was higher in *E. isabellinus*' echolocation calls compared to *E. serotinus*' calls with some overlap between 23.4 and 28.8 kHz. *E. isabellinus* showed shorter mean Dur than *E. serotinus* with an overlap between 5.0 and 16.3 ms (see Table 1 for descriptive statistics of the two species for the analysed acoustic variables).

The stepwise discriminant analysis generated one discriminant function, with PeakF and Dur as statistically significant variables.

Discriminant function (eigenvalue=0.781):

Species=1.780 (SquareRoot_Dur)+1.186 (PeakF).

Once composed of only two significant variables, a single discriminant function is defined similarly by PeakF and Dur. This function discriminates significantly the two species (Λ=0.562; $X^2_{(2)}$=28.270; p<0.001). The results of Classification Statistics showed that 27 of 34 calls from *E. isabellinus* (79.4 %) and 14 of 18 calls from *E. serotinus* (77.8 %) were classified correctly. The percentage of individuals classified correctly was 78.8 %.

Classification functions:

E. isabellinus=79.209 (SquareRoot_Dur)+18.822 (PeakF)—387.345

E. serotinus=75.663 (SquareRoot_Dur)+17.639 (PeakF)—345.053

The detailed analysis of significant variables shows that *E. isabellinus* can usually be distinguished from its counterpart for PeakF higher than 28.8 kHz. Also, pulses shorter than 5.0 ms seem to be unique for *E. isabellinus* under our recording conditions (Fig. 1). Spectrograms and oscillograms of representative calls of each species are available in Fig. 1.

Discussion

The analyses of the echolocation characteristics of Iberian *Eptesicus* species have allowed differentiation of most calls from the two cryptic species through their acoustic variables, with a 78.8 % correct identification rate. The PeakF and Dur showed significant differences between *E. serotinus* and *E. isabellinus*. *E. isabellinus* can usually be distinguished from its counterpart by a PeakF higher than 28.8 kHz. Also, pulses shorter than 5.0 ms seemed to be unique for *E. isabellinus*, at least in the conditions that we recorded the bats in (*E. serotinus* may use shorter calls in cluttered environment).

Several studies have described *E. serotinus*' calls (Obrist et al. 2004; Redgwell et al. 2009; Papadatou et al. 2008; Russo and Jones 2002) and *E. isabellinus*' echolocation (Benda et al. 2012; Disca et al. 2014) in various countries. Our results indicated that Iberian *E. serotinus* calls have lower frequencies and longer temporal variables than the previously studied European populations. On the other hand, our results showed that Iberian *E. isabellinus* calls have lower frequencies and longer temporal variables that the previously studied

Table 1 Descriptive statistics of the echolocation calls of *Eptesicus serotinus* and *E. isabellinus*

	FMax	FMin	PeakF	FMid_Pulse	Slope_Mid_Pulse	Dur	IPI
E. isabellinus (*N*=136 pulses)	52.0	24.0	29.5	30.3	1.2	7.3	126.9
	20.4–65.5	18.5–28.0	23.4–35.8	22.4–36.5	0–12.2	3.3–22.5	59.5–263.0
E. serotinus (*N*=72 pulses)	33.5	21.0	24.8	25.0	0.8	9.75	155.1
	28.5–60.0	19.0–24.5	22.7–28.8	22.8–31.2	0.2–3.9	5.0–16.3	96.8–291.0

Medians are shown above range

FMAx maximum frequency (kHz), *FMin* minimum frequency (kHz), *PeakF* peak frequency (kHz), *FMid_pulse* frequency middle pulse (kHz), *Slope_Mid_Pulse* slope (kHz/ms), *Dur* duration (ms), *IPI* inter-pulse interval (ms)

Moroccan populations, which might be the consequence of regional variations. In fact, geographical variation across Europe, as described for other bat species around the world, results from a combination of evolutionary and environmental factors (Law et al. 2002; O'Farrell et al. 2000). Jiang et al. (2013) suggesting that regional differences in calls of some

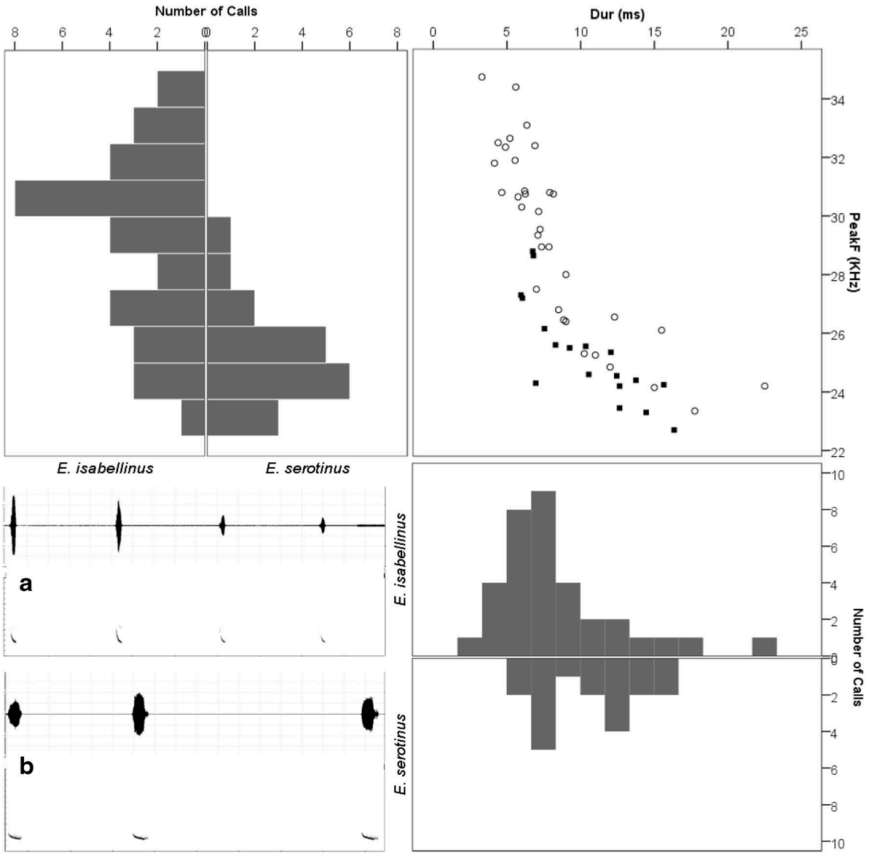

Fig. 1 Scatter plot and histogram of the significant variables that may distinguish *E. serotinus* (■) and *E. isabellinus* (○) (PeakF and Dur) (medians of the four pulses measured in each individual echolocation call). Spectrograms (*y*-axis=0–150 kHz; *x*-axis=900 ms; 50 ms/div) and oscillograms (*y*-axis=−100–100 %) of representative calls of (*a*) *E. isabellinus* and (*b*) *E. serotinus*) (FFT size 512; Hanning window—*right*) (*lower left section*). Variables' acronyms may be found in Table 1

Verpertilionidae species are not associated with morphological difference, genetic structure or geographic distance between regions. Instead, there is evidence that geographic variation in echolocation call design may have evolved as a consequence of local adaptation to climate or habitat conditions resulting in a differentiation of the ecological niches (Jones and Teeling 2006). On the other hand, perhaps for species recognition and facilitation of intraspecific communication, sympatric species tend to diverge in echolocation calls compared with allopatric populations (Russo et al. 2007).

Acoustic and ecological differences probably stem from the evolutionary history of both species. In the Iberian Peninsula, *E. serotinus* is mainly distributed in the Eurosiberian biogeographical region (more cold and humid) while *E. isabellinus* is present on the Mediterranean biogeographical region (more hot and dry) (Santos et al. 2014). Local adaptations to different climate conditions may explain some acoustic differences (Jiang et al. 2013), and the ecological niches explored by each species may be associated with different foraging habitats. Species using more cluttered sites, like dense forests, usually echolocate using a larger range of frequencies (frequently with more frequency-modulated calls) and emit shorter pulses (Neuweiler 1989). We took care to record bats in similar situations and hence minimized any variation that may be related to habitat features such as the amount of clutter.

Other factors may contribute to shaping the echolocation characteristics of these two sibling species in Iberia. Morphology probably plays a role, with larger species (within a clade) usually echolocating at lower frequencies (Bogdanowicz et al. 1999), using longer IPIs and longer call durations (Jones 1999). Our results support this pattern with the larger *E. serotinus* echolocating at lower frequencies and with longer IPI and duration than the smaller *E. isabellinus*.

Other species similar in size to *Eptesicus* use low frequency echolocation to detect relatively large prey (Jones 1995). Although *E. serotinus* is known to mainly feed on chafer and other beetles, ichneumonids and moths, large numbers of small dipterans are also present in its diet (Robinson and Stebbings 1993). *E. isabellinus* uses higher frequencies and has a more sharply frequency-modulated call, so it may be able to detect smaller prey. Consequently, it is possible that differences in the calls of these cryptic species may reflect differences in their diets. Further studies are needed to determine how diet is in fact a determinant driver of *Eptesicus* echolocation call differences in Iberia.

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